that patients with mutations in the N-terminal, transmembrane nonpore, and C-terminal regions have a significantly decreased incidence of cardiac events than those with missense mutations in the pore region (S5-loop-S6), which appear to cause dominant negative effects.[8,9]

In the present study, we identified a novel missense mutation in the transmembrane nonpore region of the *KCNH2* gene that resulted in an amino acid substitution of threonine for proline acid at position 473 (T473P) in 2 members of a Japanese family with LQTS. The proband and his father showed significantly prolonged corrected QT (QTc) interval and torsades de pointes (TdP). The electrophysiological study showed that the T473P genetic change was a dominant negative mutation that led to loss of *KCNH2* function, and Western blot analysis indicated that this mutant had a trafficking defect.

Methods

DNA isolation and mutation analysis

Genomic DNA was isolated from the subjects' white blood cells by using conventional methods and was amplified by using standard polymerase chain reaction. All exons of the *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*, and *KCNJ2* genes were sequenced by using an ABI PRISM 310 Genetic Analyzer.

Plasmid constructs and electrophysiology

The hERG cDNA was cloned into the mammalian expression vector pSI (Promega, Madison, WI). The T473P mutation was constructed by using an overlap extension strategy.

CHO-K1 cells were cultured and transiently transfected with wild-type (WT) hERG (1 μg) alone, hERG WT and hERG T473P (1 μg each), or hERG T473P (1 μg) alone by using FuGENE 6 Transfection Reagent. Cells were also cotransfected with an appropriate amount of the green fluorescent protein cloned into the pCGI vector for a total of 3 μg of cDNA per transfection. Cells displaying green fluorescence 48–72 hours after transfection were subjected to electrophysiological analysis. To test whether LQT2 mutations undergo pharmacological rescue, E4031 or thapsigargin (Alomone Labs, Jerusalem, Israel) was added to the culture media before the experimental study.

Membrane currents were studied essentially as described previously.[10] Data were acquired by using pCLAMP software (version 8.2; Axon Instruments/Molecular Devices, Sunnyvale, CA). Pooled data were expressed as mean ± standard error, and statistical comparisons were made (Origin 8.6, OriginLab, Northampton, MA) with $P < .05$ considered as significant.

Western blot analysis

Western blotting was performed as described previously.[11,12] Briefly, 2 days posttransfection, whole-cell lysates were prepared as described previously[11] by using a lysis buffer containing 50 mM Tris, 150 mM NaCl, 0.25% Triton X-100, 5 mM NaF, and protease inhibitors. Ten micrograms of cell extracts was loaded into a 7% polyacrylamide gel and prepared for polyacrylamide gel electrophoresis and Western Blot analysis. Following transfer onto nitrocellulose membranes, rabbit anti-hERG primary antibody (Alomone Labs; 1:400) and HRP-linked donkey anti-rabbit secondary antibody (Amersham Biosciences/GE Healthcare Life Sciences, Uppsala, Sweden; 1:10,000) were applied, and the bands were visualized with ECL (Amersham Biosciences/GE Healthcare Life Sciences).

Results

Clinical characterization and genetic analysis

The proband (Figure 1E, arrow) was a 37-year-old man who had syncope and was diagnosed with epilepsy at the age of 7. He had been treated with 150 mg of phenytoin; however, syncope still occurred several times a year. An electrocardiogram (ECG) taken during a routine health checkup at the age of 37 showed a mild prolongation of the QTc interval of 455 ms (Figure 1A, top). Early one morning, approximately 1 month after the health examination, the patient suddenly experienced cardiopulmonary arrest and was found to have ventricular fibrillation by the emergency crew. He received electrical defibrillation twice with an automated external defibrillator and was brought to the emergency department of our hospital. He had a significantly prolonged QTc interval of 566 ms upon admission (Figure 1A, middle). After admission, he experienced repeated TdP (Figure 1A, bottom). Temporary transvenous ventricular pacing was initiated to prevent pauses that may trigger TdP, and beta-blocker was also administered for symptomatic LQTS. He received an implantable cardioverter-defibrillator on his 21st day of hospitalization. Phenytoin was discontinued after hospital admission since his syncope was considered to be unrelated to epilepsy. He had not experienced syncope since the initiation of beta-blocker therapy.

The proband's father was also observed to have a prolongation of QTc interval at 526 ms during a family study for LQTS (Figure 1B, top) but did not have a history of syncope at that time. He had been treated with bisoprolol, valsartan, and amlodipine for hypertension since his early 60s. At the age of 74, he was afflicted with a respiratory tract infection and hypokalemia (2.9 mEq/L) as indicated by a blood test. He was prescribed 400 mg of garenoxacin once daily for the respiratory infection and subsequently developed syncope during the night. The ECG upon admission showed significant prolongation of QTc (668 ms) (Figure 1B, middle), and he experienced repeated TdP after admission (Figure 1B, bottom). He promptly discontinued the garenoxacin treatment, and instead received a replacement of potassium and temporary transvenous ventricular pacing. Bisoprolol was continued after this cardiac event. One morning, at the age of 75, he suffered a fatal cardiopulmonary arrest. Since hypokalemia (3.0 mEq/L) had been detected again during an outpatient visit a few days earlier, it is likely that TdP and ventricular fibrillation may have led to this fatal event.

Genetic analysis was performed after obtaining written informed consent, which revealed that both the proband and his father had a missense mutation consisting of an A to C

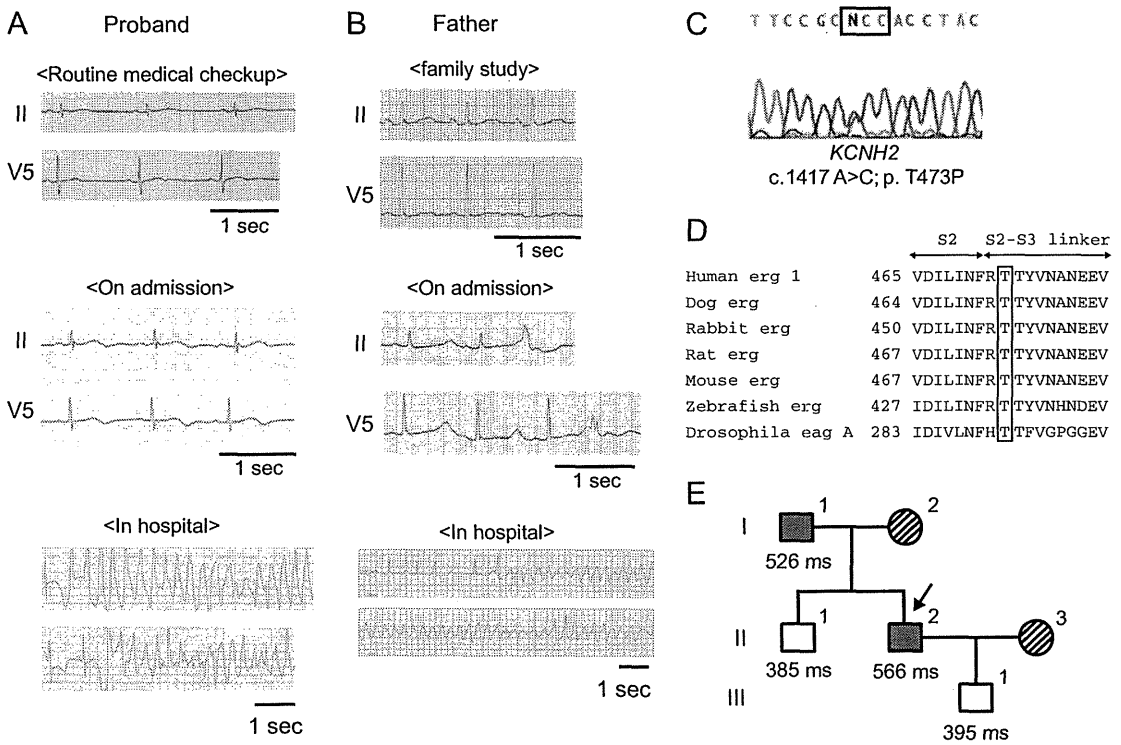

Figure 1 Electrocardiography and genetic analysis. **A:** Electrocardiograms (ECGs) of the proband. ECGs showed a mildly prolonged corrected QT (QTc) interval of 455 ms at a routine medical checkup and a significantly prolonged QTc interval of 566 ms upon hospital admission. Electrocardiographic tracing during the hospital stay showed torsades de pointes. **B:** ECGs of the proband's father. ECGs showed a prolonged QTc interval of 526 ms during a family study and a significantly prolonged QTc interval of 668 ms upon hospital admission. Electrocardiographic tracing during the hospital stay showed torsades de pointes. **C:** DNA sequence analysis of the *KCNH2* gene in the proband. A single nucleotide transition from A to C at nucleotide position 1417 in the *KCNH2* gene occurred in 2 affected family members. **D:** Amino acid sequences of the S2 and S2-S3 linkers of the hERG channel and 6 potassium channels. The box indicates the site of the T473P substitution. **E:** The pedigree and QTc intervals. The arrow in the pedigree indicates the proband. Numbers indicate the length of the QTc interval (in ms). Closed squares indicate heterozygous male patients with the *KCNH2* T473P mutation. Open squares indicate unaffected male patients without the *KCNH2* T473P mutation.

substitution at nucleotide 1417 of the *KCNH2* gene, resulting in an amino acid substitution from a highly conserved threonine to proline at position 473 of the Kv11.1 channel (Figures 1C–1E). This mutation is located in the transmembrane nonpore region and was not found in patients with the normal QTc interval or in the 150 healthy controls.

The proband's brother and son did not have a history of syncope and showed normal QTc interval in their ECGs (Figure 1E). Genetic analysis revealed that they did not have the T473P mutation.

Electrophysiological characteristics

To define the functional change of the T473P missense mutation, we transiently expressed hERG WT, hERG T473P, and hERG WT+hERG T473P in cultured mammalian cells for whole-cell voltage clamp measurements. Voltage clamp recording from WT showed a slowly activating outward current by step depolarizations (Figure 2A, left). By contrast, T473P did not express any functional channels (Figure 2A, center). When WT and T473P were coexpressed in CHO-K1 cells, the currents were less than one-fourth of control currents that were expected from expression of WT alone (Figure 2A, right). The current-voltage relationships for activating peak currents (Figure 2B) and tail currents (Figure 2C) were recorded during depolarizing pulses. The mean amplitude of the tail currents measured at −50 mV, after a depolarizing test pulse of +40 mV, was 10.5 ± 2.4 pA/pF for the WT+T473P channels (n = 20), which was significantly smaller than for WT-alone channels (40.5 ± 6.1 pA/pF; n = 15; $P < .05$). The T473P mutant did not generate any currents (n = 11). These results suggest that hERG T473P channels have dominant negative effects.

The amplitudes of the activating currents produced by the WT+T473P channels were too small to evaluate with the pulse protocol shown in Figure 2A. For this reason, we monitored recovery from inactivation (Figure 2D). The current density of the tail currents was −43.7 ± 10.5 pA/pF for WT+T473P (n = 15), which was significantly smaller than for WT alone (−105.5 ± 17.0 pA/pF; n = 15; $P < .05$) (Figure 2E). The normalized current-voltage relationships for tail currents of WT and WT+T473P showed that their mean $V_{1/2}$ values were −12.3 ± 0.9 mV (n = 15; slope factor 8.3 ± 0.5) and −10.9 ± 0.4 mV (n = 15; slope factor 11.0 ± 0.3), respectively (Figure 2F).

We next evaluated deactivation and steady-state inactivation of the T473P mutant channels. The fast and slow deactivation time constants showed no difference between

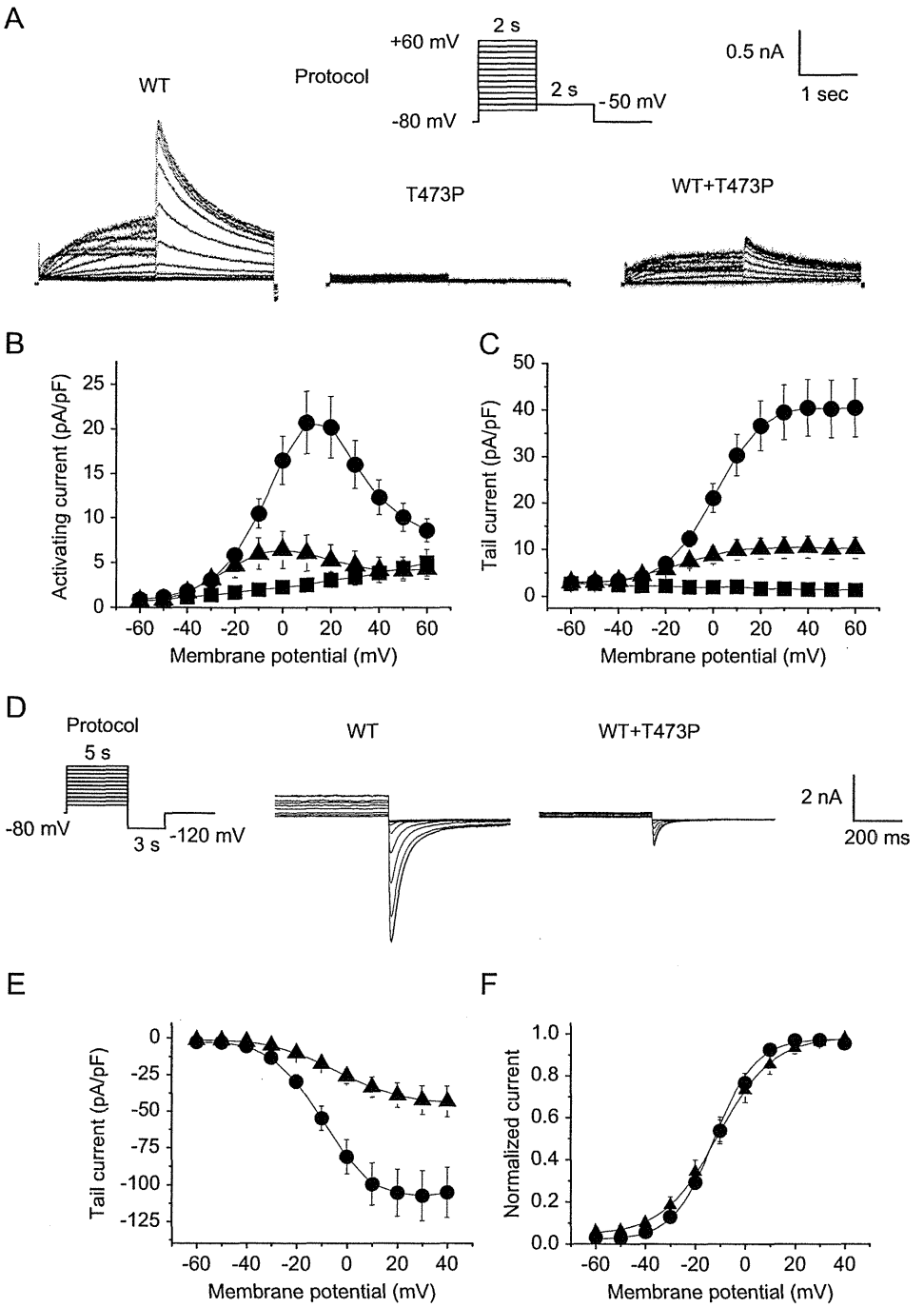

Figure 2 Functional characterization of hERG T473P in CHO-K1 cells. **A:** Representative generated currents in CHO-K1 cells, transfected with 1 μg of wild-type (WT) hERG alone (left), 1 μg of hERG T473P alone (middle), or 1 μg each of hERG WT and hERG T473P (right). Depolarizing pulses were applied from a holding potential of −80 mV to various potentials between −60 and +60 mV in 10 mV increments for 2 seconds, followed by a hyperpolarizing pulse to −50 mV for 2 seconds. **B and C:** I-V relationships for peak currents (B) and tail currents (C) in CHO-K1 cells transfected with WT alone (closed circle; n = 15), T473P (closed square; n = 11), or WT+T473P (closed triangle; n = 20). **D:** Representative generated currents in CHO-K1 cells transfected with 1 μg of WT alone or 1 μg each of WT and T473P. Currents were elicited by 5-second depolarizing pulses ranging from −60 to +40 mV, and peak tail currents were measured during a 3-second pulse to −120 mV and plotted as a function of the prepulse potential, with a holding potential of −80 mV. **E:** I-V relationships of tail currents for WT alone (closed circle; n = 15) and WT+T473P (closed triangle; n = 15). **F:** Mean amplitudes of normalized tail currents for WT alone (closed circle, n = 15) and WT+T473P (closed triangle, n = 15).

WT (n = 24) and WT+T473P channels (n = 15) (Figure 3A). Similarly, when the inactivation process was analyzed by using the voltage clamp method, the $V_{1/2}$ of inactivation yielded −56.9 ± 4.5 mV for WT (n = 20; slope factor 25.2 ± 3.3) and −59.9 ± 2.9 mV for WT+T473P (n = 20; slope factor 25.9 ± 2.1) (Figure 3B). Thus, no significant difference in the steady-state inactivation kinetics was observed as compared to WT.

Western blot analysis

To determine the effects of the T473P mutant on intracellular processing and trafficking, we assessed the glycosylation pattern by using Western blot analysis. As shown in Figure 4, WT resulted in 2 protein bands: a core-glycosylated form of about 135 kDa and a mature, fully glycosylated form of about 155 kDa. By contrast, the T473P mutant showed only 1 band of 135 kDa. This finding indicated that the T473P protein is incompletely processed and does not traffic correctly.

Effect of E4031 or thapsigargin on KCNH2 T473P mutation

Previous reports have shown that drugs that bind to hERG, such as E4031 and the SERCA inhibitor thapsigargin, can

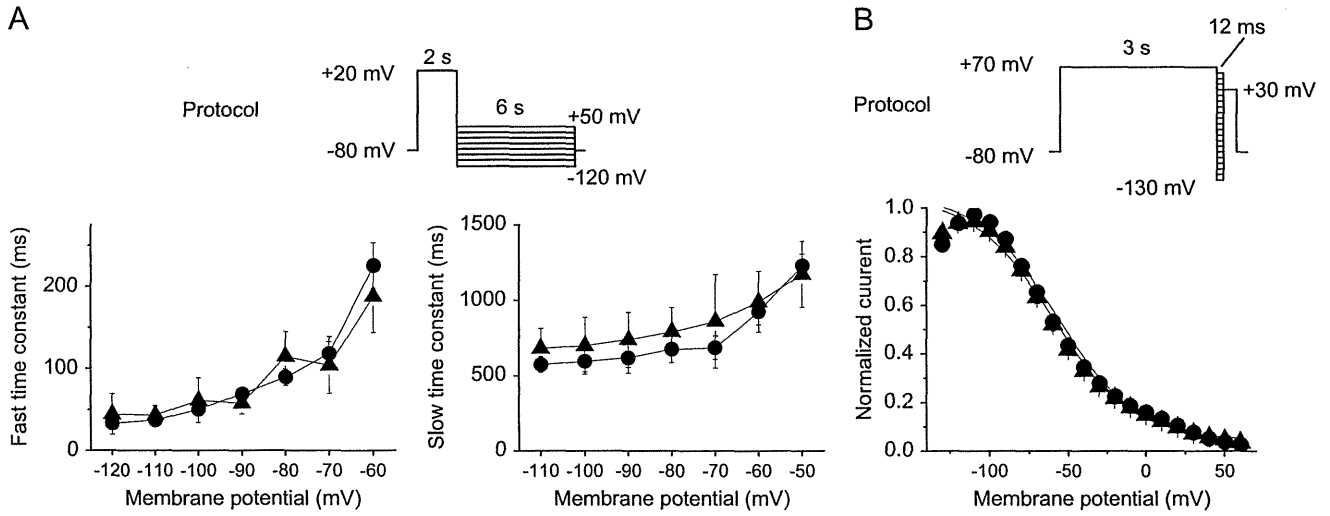

Figure 3 Deactivation and steady-state inactivation of hERG T473P in CHO-K1 cells. **A:** Deactivation time constants of wild-type (WT) (closed circle; n = 24) and WT+T473P (closed triangle; n = 15). Current was activated by 2-second pulses to +20 mV, followed by a return to test potentials between −50 and −100 mV. **B:** Normalized steady-state inactivation curves of WT (closed circle; n = 20) and WT+T473P (closed triangle; n = 20). To construct the inactivation curve, a voltage protocol (inset) was employed: a 3-second depolarizing pulse to inactivate hERG channels, followed by varying repolarizing pulses to a potential between −130 and +60 mV for 12 ms, and then a test pulse to +30 mV. The current amplitude at the test potential was normalized and plotted against the prepulse potential. Curves represent best fits to a Boltzmann function.

rescue trafficking defects of certain trafficking-deficient hERG variants[13–15]; therefore, we assessed the effects of the 2 drugs on T473P mutants. As a control, we included the hERG variant G601S, which is known to be rescued by this treatment, in our analysis. In cells expressing the trafficking-defective hERG G601S channels, the absolute peak tail current density in control condition was 19.2 ± 5.0 pA/pF (n = 17; Figure 5, left). After 24 hours of incubation with 10 μM E4031 or 1 μM thapsigargin, the current was significantly increased with an absolute peak tail current density of 58.0 ± 15.5 pA/pF (n = 10; $P < .05$) and 47.2 ± 14.6 pA/pF (n = 8; $P < .05$), respectively (Figure 5, left). Unlike the G601S-transfected cells, there was no current in cells expressing the hERG T473P channels in control condition (n = 9) (Figure 5, center). Administration of 10 μM E4031 or 1 μM thapsigargin for 24 hours resulted in no pharmacological rescue of hERG current (n = 9 and n = 8, respectively; Figure 5, center). We next studied the effect of E4031 or thapsigargin treatment on the coassembly of hERG WT and the T473P mutant (Figure 5, right). E4031 exposure significantly increased the absolute tail current density from 33.8 ± 5.7 pA/pF (n = 25) in control cells to 67.3 ± 16.0 pA/pF (n = 15; $P < .05$) in treated cells (Figure 5, right). By contrast, thapsigargin exposure did not affect *KCNH2* WT current; the absolute peak tail current density was 32.2 ± 6.9 pA/pF (n = 19) in treated cells, which was not significantly different from that in controls (Figure 5, right).

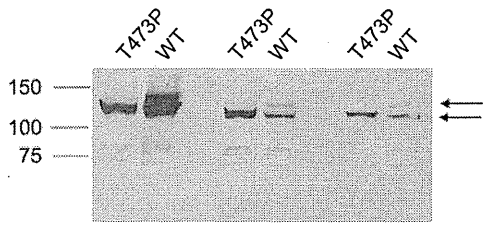

Figure 4 Incomplete glycosylation of hERG T473P indicates lack of intracellular processing and trafficking. In 3 independent experiments, hERG wild-type (WT) and hERG T473P were transfected into 100-mm dishes of CHO cells, as described previously. Two days posttransfection, whole-cell lysates were prepared as described previously by using a lysis buffer containing 50 mM Tris, 150 mM NaCl, 0.25% Triton X-100, and 5 mM NaF. Ten micrograms of cell extracts was loaded into a 7% polyacrylamide gel and prepared for polyacrylamide gel electrophoresis and Western blot analysis. Following transfer onto nitrocellulose membranes, rabbit anti-HERG 1 primary antibody (Alomone, 1:400) and HRP-linked donkey anti-rabbit secondary antibody (1:10,000, Amersham Biosciences/GE Healthcare Life Sciences) were applied. The blot was developed with ECL (Amersham Biosciences/GE Healthcare Life Sciences). Molecular weight markers are indicated on the left. The arrows on the right mark the position of the fully (top) and incompletely (bottom) glycosylated hERG proteins. In all 3 independent experiments, T473P remained incompletely glycosylated while hERG WT showed the mature glycosylation pattern.

Discussion

In this report, we describe a novel missense *KCNH2* mutation in patients who exhibited the congenital or acquired form of LQTS. The proband (Figure 1E, II-2 and arrow) was first diagnosed with epilepsy when he was 7 years old but continued to have repeated syncope in spite of continued antiepileptic medication. Previous reports indicate that patients are sometimes initially misdiagnosed with epilepsy and later receive a delayed diagnosis of LQTS.[16,17] The misdiagnosis of epilepsy and the treatment with antiepileptic drug medications is especially common in patients with LQT2.[17] We consider it likely that the index patient's repeated syncope was caused by TdP associated with LQTS, rather than epilepsy because 3 decades later he developed ventricular fibrillation and was diagnosed with LQTS. This is further supported by the fact that he did not experience

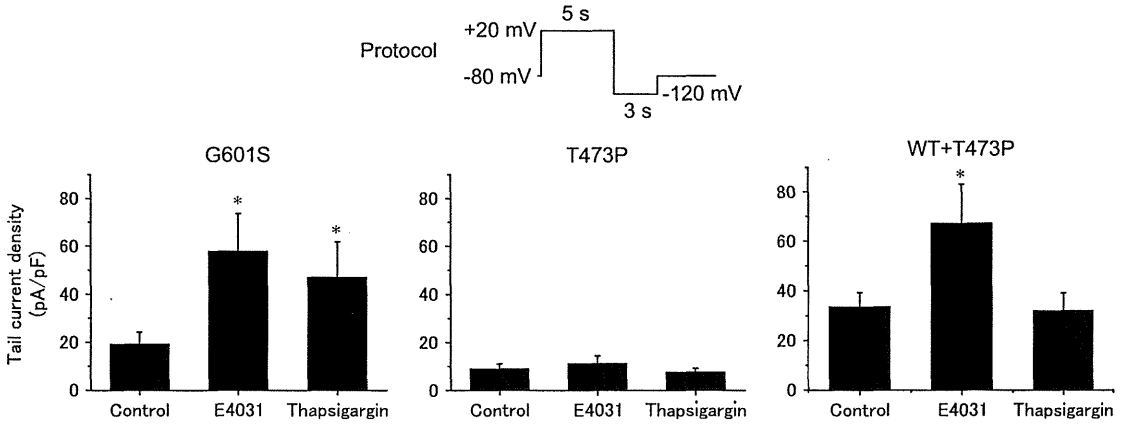

Figure 5 Effect of E4031 or thapsigargin on the trafficking-defective *KCNH2* mutations. The voltage clamp protocol is shown in the inset. From a holding potential of −80 mV, 5-second depolarizing pulses of +20 mV were applied, followed by a 3-second pulse of −120 mV. Absolute peak tail current densities of the G601S current, the T473P current, or the coexpressed WT+T473P current recorded at −120 mV from control cells, cells incubated in 10 μM of E4031 for 24 hours, or cells incubated in 1 μM of thapsigargin for 24 hours. $^{*}P < .05$ vs control cells.

syncope after the initiation of beta-blocker therapy. The proband's ECGs showed diurnal variability in the QTc interval, which might have resulted in his delayed diagnosis of LQTS; the QTc interval taken during daytime 1 month before the serious arrhythmic event was not significantly prolonged. According to previous reports, QTc intervals are longer at night than during the day in normal subjects.[18] The QTc interval and variability peak shortly after awakening, which may reflect increased autonomic instability and explain the increased vulnerability to ventricular tachycardia and sudden cardiac death in the morning.[18] Diurnal variability in QTc interval duration in LQTS has also been shown. Patients with LQT1 and LQT2 show trends for modest QTc shortening and lengthening, respectively, during the night compared with daytime, while patients with LQT3 show clear lengthening of the QTc interval during the night.[19] Goldenberg et al[20] reported that there was considerable variability in QTc measures in serial follow-up ECGs, and the maximum QTc interval provided incremental prognostic information in LQTS. Forty-one percent of the study patients had a maximum QTc of >500 ms, whereas only 25% of the patients had a baseline QTc interval of >500 ms during adolescence.[20]

The proband's father showed prolonged QTc interval and developed repeated TdP under hypokalemic conditions and after the administration of garenoxacin. Generally, the QT interval is prolonged by low extracellular potassium, which decreases I_{Kr} by enhancing I_{Kr} inactivation,[21] accelerating internalization and degradation of hERG channels,[22] and enhancing blockage of the hERG channels by extracellular sodium.[23] Some fluoroquinolones inhibit I_{Kr} and have been associated with TdP, resulting in QTc interval prolongation.[24] Garenoxacin, a novel quinolone antibiotic agent, is reportedly safe in healthy subjects.[25] However, in a separate report, TdP was induced by adding oral garenoxacin to disopyramide under hypokalemic conditions.[26] In this particular case, it was possible that reduced repolarization reserves due to enhanced I_{Kr} inhibition caused by a combination of *KCNH2* mutation, hypokalemia and oral garenoxacin contributed to QT interval prolongation and arrhythmia development.[27]

We identified a novel missense mutation in the transmembrane nonpore region of the hERG protein from patients with LQTS. Most mutations involving the pore region of *KCNH2* are missense mutations with dominant negative effects, whereas those in the nonpore regions are mostly associated with coassembly or trafficking abnormalities resulting in haplotype insufficiency.[28] A previous report showed that patients with missense mutations in the transmembrane nonpore region did not have significantly higher rates of cardiac events as compared to patients with missense mutations in the transmembrane pore region.[9] The *KCNH2* T473P mutation identified in this study showed trafficking abnormality and generated no current at all while exhibiting dominant negative effects on WT channels. Patients harboring this mutation showed a severe clinical course. The finding of a nonpore mutation that can cause a dominant negative effect is not novel: mutations in the *KCNH2*C-terminal region, A915fs+47X and G816V, also caused a trafficking defect that acted in a partially dominant negative manner.[29,30] In addition, previous studies reported several mutations that are located adjacent to position 473 of the *KCNH2* gene.[15,31–33] Interestingly, *KCNH2* D456Y,[15] F463L,[31] N470D,[15,32] and T474I[15,33] mutants all displayed protein trafficking deficiencies and caused a dominant negative effect on the WT hERG current. To explain this, a previous study proposed that the misfolded mutant subunits assemble with WT subunits in the endoplasmic reticulum to cause endoplasmic reticulum retention of the coassembled channels by the quality control system.[34]

Many trafficking-deficient LQT2 channels can be pharmacologically rescued by the administration of the drug E4031, which causes high-affinity inhibition of hERG channels, or the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin.[13–15] In the electrophysiological study, homomeric T473P channels were not pharmacologically rescued by

either E4031 or thapsigargin (Figure 5, center). Consistent with these findings, our preliminary study showed that despite a 6-hour treatment with 1 μM thapsigargin, complete glycosylation of hERG T473P was still not observed on Western blot. However, our electrophysiological study showed that heteromeric channels (coassembled hERG WT and T473P subunits) were rescued only by E4031 (Figure 5, right). Thus, this indicated that T473P is partially and selectively rescued by E4031. A previous study showed that E4031 was also able to pharmacologically rescue the mutations adjacent to position 473 both upstream (N470D) and downstream (T474I), although thapsigargin could not.[15] This region may include trafficking-deficient mutations that can be rescued by E4031, but not by thapsigargin.

Our study identified a novel genetic change in the *KCNH2* gene, which indicated that certain mutations in the transmembrane nonpore region can result in protein trafficking defects and exhibit dominant negative effects. These mutations seem to be concentrated in the region between S2 and S2-S3 linkers of the hERG channel and may cause severe clinical course in affected patients.

References

1. Moss AJ, Schwartz PJ, Crampton RS, et al. The long QT syndrome: prospective longitudinal study of 328 families. Circulation 1991;84:1136–1144.
2. Napolitano C, Bloise R, Monteforte N, Priori SG. Sudden cardiac death and genetic ion channelopathies: long QT, Brugada, short QT, catecholaminergic polymorphic ventricular tachycardia, and idiopathic ventricular fibrillation. Circulation 2012;125:2027–2034.
3. Roden DM, Viswanathan PC. Genetics of acquired long QT syndrome. J Clin Invest 2005;115:2025–2032.
4. Splawski I, Shen J, Timothy KW, et al. Spectrum of mutations in long-QT syndrome genes: *KVLQT1*, *HERG*, *SCN5A*, *KCNE1*, and *KCNE2*. Circulation 2000;102:1178–1185.
5. Hayashi K, Shimizu M, Ino H, et al. Characterization of a novel missense mutation *E637K* in the pore-S6 loop of HERG in a patient with long QT syndrome. Cardiovasc Res 2002;54:67–76.
6. Hayashi K, Shimizu M, Ino H, et al. Probucol aggravates long QT syndrome associated with a novel missense mutation M124T in the N-terminus of HERG. Clin Sci (Lond) 2004;107:175–182.
7. Itoh H, Sakaguchi T, Ding WG, et al. Latent genetic backgrounds and molecular pathogenesis in drug-induced long-QT syndrome. Circ Arrhythm Electrophysiol 2009;2:511–523.
8. Moss AJ, Zareba W, Kaufman ES, et al. Increased risk of arrhythmic events in long-QT syndrome with mutations in the pore region of the human ether-a-go-go-related gene potassium channel. Circulation 2002;105:794–799.
9. Shimizu W, Moss AJ, Wilde AA, et al. Genotype-phenotype aspects of type 2 long QT syndrome. J Am Coll Cardiol 2009;54:2052–2062.
10. Hayashi K, Fujino N, Ino H, et al. A KCR1 variant implicated in susceptibility to the long QT syndrome. J Mol Cell Cardiol 2011;50:50–57.
11. Kupershmidt S, Yang IC, Hayashi K, et al. The IKr drug response is modulated by KCR1 in transfected cardiac and noncardiac cell lines. FASEB J 2003;17: 2263–2265.
12. Nakajima T, Hayashi K, Viswanathan PC, et al. HERG is protected from pharmacological block by alpha-1,2-glucosyltransferase function. J Biol Chem 2007;282:5506–5513.
13. Zhou Z, Gong Q. January CT. Correction of defective protein trafficking of a mutant HERG potassium channel in human long QT syndrome: pharmacological and temperature effects. J Biol Chem 1999;274:31123–31126.
14. Delisle BP, Anderson CL, Balijepalli RC, et al. Thapsigargin selectively rescues the trafficking defective LQT2 channels G601S and F805C. J Biol Chem 2003;278:35749–35754.
15. Anderson CL, Delisle BP, Anson BD, et al. Most LQT2 mutations reduce Kv11.1 (hERG) current by a class 2 (trafficking-deficient) mechanism. Circulation 2006;113:365–373.
16. MacCormick JM, McAlister H, Crawford J, et al. Misdiagnosis of long QT syndrome as epilepsy at first presentation. Ann Emerg Med 2009;54:26–32.
17. Johnson JN, Hofman N, Haglund CM, et al. Identification of a possible pathogenic link between congenital long QT syndrome and epilepsy. Neurology 2009;72:224–231.
18. Molnar J, Zhang F, Weiss J, Ehlert FA, Rosenthal JE. Diurnal pattern of QTc interval: how long is prolonged? Possible relation to circadian triggers of cardiovascular events. J Am Coll Cardiol 1996;27:76–83.
19. Kaufman ES, Priori SG, Napolitano C, et al. Electrocardiographic prediction of abnormal genotype in congenital long QT syndrome: experience in 101 related family members. J Cardiovasc Electrophysiol 2001;12:455–461.
20. Goldenberg I, Mathew J, Moss AJ, et al. Corrected QT variability in serial electrocardiograms in long QT syndrome: the importance of the maximum corrected QT for risk stratification. J Am Coll Cardiol 2006;48:1047–1052.
21. Yang T, Snyders DJ, Roden DM. Rapid inactivation determines the rectification and $[K^+]_o$ dependence of the rapid component of the delayed rectifier K^+ current in cardiac cells. Circ Res 1997;80:782–789.
22. Guo J, Massaeli H, Xu J, et al. Extracellular K^+ concentration controls cell surface density of IKr in rabbit hearts and of the HERG channel in human cell lines. J Clin Invest 2009;119:2745–2757.
23. Numaguchi H, Johnson JP, Jr, Petersen CI, Balser JR. A sensitive mechanism for cation modulation of potassium current. Nat Neurosci 2000;3:429–430.
24. Kang J, Wang L, Chen XL, Triggle DJ, Rampe D. Interactions of a series of fluoroquinolone antibacterial drugs with the human cardiac K^+ channel HERG. Mol Pharmacol 2001;59:122–126.
25. Wang Z, Grasela DM, Krishna G. Retrospective analysis of electrocardiographic changes after administration of oral or intravenous garenoxacin in five phase I, placebo-controlled studies in healthy volunteers. Clin Ther 2007;29:1098–1106.
26. Miyamoto K, Kawai H, Aoyama R, et al. Torsades de pointes induced by a combination of garenoxacin and disopyramide and other cytochrome P450, family 3, subfamily A polypeptide-4-influencing drugs during hypokalemia due to licorice. Clin Exp Nephrol 2010;14:164–167.
27. Roden DM, Long QT. syndrome: reduced repolarization reserve and the genetic link. J Intern Med 2006;259:59–69.
28. January CT, Gong Q, Zhou Z. Long QT syndrome: cellular basis and arrhythmia mechanism in LQT2. J Cardiovasc Electrophysiol 2000;11:1413–1418.
29. Christe G, Theriault O, Chahine M, et al. A new C-terminal hERG mutation A915fs+47X associated with symptomatic LQT2 and auditory-trigger syncope. Heart Rhythm 2008;5:1577–1586.
30. Krishnan Y, Zheng R, Walsh C, Tang Y, McDonald TV. Partially dominant mutant channel defect corresponding with intermediate LQT2 phenotype. Pacing Clin Electrophysiol 2012;35:3–16.
31. Yang HT, Sun CF, Cui CC, et al. HERG-F463L potassium channels linked to long QT syndrome reduce I(Kr) current by a trafficking-deficient mechanism. Clin Exp Pharmacol Physiol 2009;36:822–827.
32. Sanguinetti MC, Curran ME, Spector PS, Keating MT. Spectrum of HERG K^+-channel dysfunction in an inherited cardiac arrhythmia. Proc Natl Acad Sci U S A 1996;93:2208–2212.
33. Nakajima T, Furukawa T, Tanaka T, et al. Novel mechanism of HERG current suppression in LQT2: shift in voltage dependence of HERG inactivation. Circ Res 1998;83:415–422.
34. Gong Q, Anderson CL. January CT, Zhou Z. Pharmacological rescue of trafficking defective HERG channels formed by coassembly of wild-type and long QT mutant N470D subunits. Am J Physiol Heart Circ Physiol 2004;287: H652–H658.

Clinical Investigations

Impact of Systolic Dysfunction in Genotyped Hypertrophic Cardiomyopathy

Address for correspondence:
Noboru Fujino, MD
Division of Cardiovascular Medicine
Kanazawa University Graduate
School of Medical Science
Takara-machi 13-1
Kanazawa-City,
Ishikawa-Prefecture, Japan
nfujino@mhs.mp.kanazawa-u.ac.jp

Noboru Fujino, MD; Tetsuo Konno, MD; Kenshi Hayashi, MD; Akihiko Hodatsu, MD; Takashi Fujita, MD; Toyonobu Tsuda, MD; Yoji Nagata MD; Masa-aki Kawashiri, MD; Hidekazu Ino, MD; Masakazu Yamagishi, MD
Division of Cardiovascular Medicine, Kanazawa University Graduate School of Medical Science, Kanazawa-City, Japan

ABSTRACT

Background: Hypertrophic cardiomyopathy (HCM) is a disease of the sarcomere, and approximately 5% of cases of HCM show systolic dysfunction with poor prognosis. Few data exist regarding the systolic dysfunction in a large population of genotyped HCM subjects.

Hypothesis: The aim of this study was to assess the systolic dysfunction and prognosis in sarcomere gene mutation carriers.

Methods: The study included 157 sarcomere gene mutation carriers from 69 unrelated HCM families (87 males; mean age, 46.5 ± 20.5 years). After exclusions for systolic dysfunction at baseline, 107 subjects underwent serial echocardiograms.

Results: At a mean follow-up of 7.0 years, 12 subjects experienced systolic dysfunction. In multivariate Cox analysis, systolic dysfunction was related to age and ejection fraction at initial evaluation ($P < 0.001$ and $P = 0.020$, respectively), and was associated with the absence of mutations in the cardiac myosin-binding protein C gene (*MYBPC3*) ($P = 0.042$). When the subjects were divided into *MYBPC3* and non-*MYBPC3* mutation carriers, and time from birth to development of systolic dysfunction was compared, the rate of systolic dysfunction was higher in the non-*MYBPC3* group than in *MYBPC3* group (Kaplan-Meier, log-rank test, $P = 0.010$). After the onset of systolic dysfunction, 11 of 12 subjects died during a mean follow-up of 8.3 years.

Conclusions: Non-*MYBPC3* mutation carriers developed left ventricular systolic dysfunction more frequently than *MYBPC3* mutation carriers, and the majority of sarcomere gene mutation carriers with systolic dysfunction had fatal outcomes during follow-up. This suggests that subjects with mutations in sarcomeric genes require careful management for systolic dysfunction.

Introduction

Hypertrophic cardiomyopathy (HCM) is a primary disorder of the myocardium that causes distinctive anatomic and histologic features,[1] and it is the most frequent cause of sudden cardiac death in young athletes.[2] Epidemiologic data indicate that 2 in 1000 young adults have unexplained hypertrophy.[3] Mutations in genes that encode sarcomere proteins including cardiac myosin-binding protein C gene (*MYBPC3*), cardiac troponin T gene (*TNNT2*), cardiac troponin I gene (*TNNI3*), and cardiac β-myosin heavy chain gene (*MYH7*) are well-established causes of the disease.[1,4] Studies in populations with familial HCM often reported that mutations in sarcomere proteins could be detected in around 50% of study subjects.[4–6] Left ventricular systolic dysfunction has been regarded as a relatively common disease complication of HCM.[7–10] However, the characterization of left ventricular systolic dysfunction has been hindered by the small number of subjects with the disease (single cases or small groups of subjects).[11–13] We have reported that subjects with HCM caused by mutations in *TNNT2* and *TNNI3* start to develop left ventricular systolic dysfunction at around 40 years of age.[14–17] In addition, we and others have reported that subjects with HCM caused by mutations in *MYBPC3* also progress to left ventricular systolic dysfunction,[18,19] although the clinical features of HCM associated with mutations in *MYBPC3* have late onset and a favorable clinical course.[20] These data may provide useful information on genetic counseling strategies of affected subjects with sarcomere gene mutations. However, these previous studies have been based largely on HCM subjects with a single gene mutation.[14–19]

The primary aim of the present study was to compare the clinical course of left ventricular systolic dysfunction

This work was supported in part by a Grant-in-Aid for Scientific Research, the Ministry of Education, Culture, Sports, Science, and Technology (KAKENHI 16790414, 19590807, 22590808, Tokyo, Japan), and the Research Grant for Cardiovascular Diseases (20C-4) from the Ministry of Health, Labour, and Welfare (Tokyo, Japan).
The authors have no other funding, financial relationships, or conflicts of interest to disclose.

Received: September 4, 2012
Accepted with revision: October 31, 2012

Published online in Wiley Online Library (wileyonlinelibrary.com)
DOI:10.1002/clc.22082

in subjects with HCM in a relatively large population with mutations in various sarcomeric genes. The secondary aim was to assess the prognosis of the HCM subjects who developed left ventricular systolic dysfunction.

Methods

This study included 69 unrelated probands with HCM exhibiting disease-causing mutations in genes such as *MYBPC3*, *TNNT2*, *TNNI3*, and *MYH7*. In addition, their family members were evaluated clinically and genetically, and 88 carriers with the same etiological sarcomere gene mutation as each proband were identified. Thus, a total 157 genetically affected subjects (87 males; mean age, 46.5 ± 20.5 years) comprised the study population. All subjects were identified at Kanazawa University Hospital or its affiliated hospitals (from primary to tertiary care centers) between 1998 and 2009. The diagnosis of HCM was based on the echocardiographic demonstration of left ventricular hypertrophy (LVH) (maximal left ventricular wall thickness ≥13 mm) in the absence of other cardiac or systemic causes for the left ventricular hypertrophy. These subjects also met the definition and classification proposed by the 1995 World Health Organization/International Society and Federation of Cardiology Task Force.[21] Subjects with systolic dysfunction were also included in this study. To compare the differences in the clinical course between several disease-causing genes, carriers with multiple mutations were not included in this study. Written informed consent was obtained from all subjects or from the parents of minors participating in the study in accordance with the guidelines of the Bioethical Committee on Medical Research, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan.

DNA was isolated from peripheral white blood cells of all subjects by use of a DNA extractor (ABI 341 Genepure Nucleic Acid Purification System; Applied Biosystems, Carlsbad, CA). In vitro amplification of genomic DNA was performed via polymerase chain reaction (PCR). Oligonucleotide primers were used to amplify all exons and exon-intron boundaries of 4 sarcomere genes, namely *MYBPC3*, *TNNT2*, *TNNI3*, and *MYH7* using standard protocols as previously described.[14–18] Single-strand conformational polymorphism analysis of amplified DNA was then performed as previously described,[14–18] with a slight modification. We also screened for mutations in sarcomere genes using high-resolution melt analysis as previously described.[22] For abnormal single-strand conformational polymorphism patterns or abnormal melt profiles, the nucleotide sequences of the cloned PCR products were determined on both strands (bidirectional sequencing) by the dye terminator cycle sequencing method using an automated fluorescent sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). The sequence variation was confirmed by restriction enzyme digestion. Samples from 400 chromosomes of 200 ethnicity-matched normal individuals were also analyzed.

Standard transthoracic M-mode and 2-dimensional echocardiographic studies were performed to identify and quantify the morphological features of the left atrium and left ventricle. The left atrial dimension was measured at end-systole. Maximal wall thickness (MWT) of the left ventricle was defined as the greatest thickness in any single segment. Left ventricular end-diastolic dimension (LVDd), left ventricular end-systolic dimension (LVDs), interventricular septal thicknesses (IVST), and posterior wall thickness (PWT) were measured at the level of the tips of the mitral valve leaflets. Ejection fraction (EF) was calculated by Teicholz's method and by modified Simpson's method when left ventricular dilatation or regional decrease of left ventricular wall motion occurred. Systolic dysfunction was defined as EF <50% according to a previous report.[23] The echocardiographic parameters and the course of development of systolic dysfunction were compared among the genotyped HCM subjects. Differences between values measured at baseline and follow-up were analyzed by the Student paired *t* test. Differences between groups were analyzed by the Student unpaired *t* test. Categorical variables were compared by the χ^2 test for independent variables. A multivariate Cox analysis was performed to find predictor factors of systolic dysfunction. The age that subjects developed systolic dysfunction for the first time was estimated according to the Kaplan-Meier method, and comparison of the 2 groups was performed by means of the log-rank test. A P value <0.05 was considered statistically significant in all analyses. Statistical analyses were carried out with the computer software SPSS version 17.0 (IBM SPSS, Armonk, NY).

Results

The clinical and genetic characteristics of the study population are presented in Table 1; 26 different mutations were identified in 157 subjects. Left ventricular outflow tract obstruction (pressure gradient at rest >30 mm Hg) was detected only in 4 subjects (hypertrophic obstructive cardiomyopathy), and none of them underwent percutaneous septal ablation. Most of these mutations have been identified and described elsewhere.[14–19,24–27] Three nonsense mutations, p.Gln541ter, p.Tyr816ter, and p.Gln827ter in *MYBPC3* and 3 missense mutations, p.Ala200Thr, p.Ala321Val, and p.Ser866Pro in *MYH7* were novel and presumed to be pathogenic by standard criteria of the absence of the mutation in large numbers of normal controls, alteration of evolutionarily conserved residues, and/or predicted impact on protein structure. Two missense mutations p.Val85Leu in *TNNT2* and the missense mutation p.Met822Leu in *MYH7* were due to alteration in the nucleotide sequences, GTG to TTG, ATG to CTG, respectively; this the first report of these nucleotide alterations in these genes.

At the initial evaluation, 9 subjects showed systolic dysfunction. Of the 9 subjects, 3 were *MYBPC3* mutation carriers (2 of p.Arg820Gln from 2 families and 1 of p.Gln998Glu), 2 were *TNNT2* mutation carriers (both p.Arg92Trp from 1 family), 2 were *TNNI3* mutation carriers (both p.Lys183del from 2 families), and 2 were *MYH7* mutation carriers (1 of p.Gly733Glu and 1 of p.Met822Leu). As for the frequencies of subjects with systolic dysfunction, 5.4% (3/56) were *MYBPC3* mutation carriers, 8.7% (2/23) were *TNNT2* mutation carriers, 3.6% (2/56) were *TNNI3* mutation carriers, and 9.1% (2/22) were *MYH7* mutation carriers. There were no differences among the carriers

Published online in Wiley Online Library (wileyonlinelibrary.com)
DOI:10.1002/clc.22082

Table 1. Clinical and Genetic Characteristics of the Study Population

No.	157
Age (y)	46.5 ± 20.5
Male (%)	87 (55.4)
Echocardiography	
LAD (mm)	37.6 ± 7.5
IVST (mm)	14.7 ± 5.4
PWT (mm)	10.5 ± 2.3
MWT (mm)	15.3 ± 5.6
LVDd (mm)	44.5 ± 6.3
LVDs (mm)	28.0 ± 7.0
EF (%)	67.1 ± 10.9
HOCM	4 (2.5%)
Disease-causing gene (%)	
MYBPC3	56 (35.7)
TNNT2	23 (14.6)
TNNI3	56 (35.7)
MYH7	22 (14.0)
Medications (%)	
Calcium channel blocker	27 (17.2)
β-Blocker	24 (15.3)
ACE-I or ARB blocker	29 (18.5)

Abbreviations: ACE-I, angiotensin-converting enzyme-inhibitor; ARB, angiotensin receptor blocker; EF, ejection fraction; HOCM, hypertrophic obstructive cardiomyopathy; IVST, interventricular septal thickness; LAD, left atrial dimension; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; MWT, maximal wall thickness; *MYBPC3*, cardiac myosin-binding protein C gene; *MYH7*, cardiac β-myosin heavy chain gene; PWT, posterior wall thickness; *TNNI3*, cardiac troponin I gene; *TNNT2*, cardiac troponin T gene. Values are mean ± SD unless otherwise shown.

Table 2. Serial Changes of Echocardiographic Parameters During Follow-up

	Baseline	*P* Value	Follow-up	*P* Value
Cases				
MYBPC3	36			
Non-*MYBPC3*	71			
Male (%)				
MYBPC3	24 (66.7)	0.017		
Non-*MYBPC3*	30 (42.3)			
Age, y				
MYBPC3	50.1 ± 19.9	0.055		
Non-*MYBPC3*	41.9 ± 21.0			
HOCM (%)				
MYBPC3	2 (5.6)	0.480		
Non-*MYBPC3*	2 (2.8)			
Medications (%)				
Calcium channel blocker				
MYBPC3	8 (22.2)	0.721		
Non-*MYBPC3*	18 (25.4)			
β-Blocker				
MYBPC3	5 (13.9)	0.287		
Non-*MYBPC3*	16 (22.5)			
ACE-I or ARB				
MYBPC3	7 (19.4)	0.598		
Non-*MYBPC3*	17 (23.9)			
IVST				
MYBPC3	15.8 ± 5.6	0.565	17.2 ± 6.0	0.118
Non-*MYBPC3*	15.1 ± 5.6		15.4 ± 5.5	
PWT				
MYBPC3	11.1 ± 2.3	0.178	10.4 ± 1.8	0.529
Non-*MYBPC3*	10.4 ± 2.6		10.2 ± 2.5	
MWT				
MYBPC3	16.6 ± 5.9	0.456	17.5 ± 6.0	0.194
Non-*MYBPC3*	15.7 ± 5.8		15.9 ± 5.9	
LVDd				
MYBPC3	43.8 ± 4.9	0.754	44.9 ± 5.8	0.717
Non-*MYBPC3*	43.5 ± 5.5		44.4 ± 7.0	

of the 4 genes with respect to the frequency of systolic dysfunction ($P = 0.72$). To study the clinical course of the development of systolic dysfunction, 9 subjects who had systolic dysfunction at the initial evaluation were excluded, and 107 of the 157 subjects underwent serial echocardiography at least a month apart. The data from 107 subjects (54 males; mean age, 44.7 ± 20.9 years; mean follow-up, 7.0 ± 4.9 years) were analyzed. During the mean follow-up period of 7 years, 12 subjects out of 107 experienced systolic dysfunction (1.60 cases per 100 person-years). In multivariate Cox analysis, systolic dysfunction was closely related to the age and ejection fraction at initial evaluation ($P < 0.001$ and $P = 0.020$, respectively), and was closely related to the absence of mutation in *MYBPC3* as a disease-causing gene ($P = 0.042$). Therefore, we divided the 107 subjects into 2 groups, those with *MYBPC3* mutations and those with other sarcomere gene mutations (non-*MYBPC3*), and compared the age to the development of systolic dysfunction.

There were 36 subjects in *MYBPC3* and 71 subjects in non-*MYBPC3* groups. At the time of initial evaluation,

Published online in Wiley Online Library (wileyonlinelibrary.com)
DOI:10.1002/clc.22082

Table 2. *Continued*

	Baseline	*P* Value	Follow-up	*P* Value
LVDs				
MYBPC3	26.5 ± 3.8	0.829	27.7 ± 5.5	0.344
Non-*MYBPC3*	26.7 ± 5.1		29.1 ± 7.9[a]	

Abbreviations: ACE-I, angiotensin-converting enzyme-inhibitor; ARB, angiotensin receptor blocker; HOCM, hypertrophic obstructive cardiomyopathy; IVST, interventricular septal thickness; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; MWT, maximal wall thickness; PWT, posterior wall thickness. Values are mean ± standard deviation unless otherwise shown. *P* values concern the comparison of the values at baseline and follow-up between *MYBPC3* and non-*MYBPC3* (12 of *TNNT2*, 46 of *TNNI3*, and 13 of *MYH7*). [a]$P < 0.05$ compared with baseline within group by paired *t* test.

no significant differences were found between *MYBPC3* and non-*MYBPC3* groups, respectively, for the following variables: IVST, PWT, MWT, LVDd, and LVDs (Table 2). There was no significant difference in EF between the 2 groups at baseline. However, the percent decrease in EF was significantly greater in the non-*MYBPC3* group between baseline and last evaluation or the time of development of systolic dysfunction (69.0 ± 8.4% vs 63.7 ± 13.0%; $P < 0.001$) (Figure 1), whereas there was no significant change in the *MYBPC3* group in this time interval. Furthermore, Kaplan-Meier analysis comparing the age at which systolic dysfunction developed showed a significant difference between *MYBPC3* and non-*MYBPC3* groups (log-rank test, $P = 0.010$) (Figure 2). In the *MYBPC3* group, 1 subject out of 36 subjects developed to systolic dysfunction at the age of 71 in 6.3 years (0.44 cases per 100 person-years). On the other hand, in the non-*MYBPC3* group, 11 subjects out of 71 subjects developed to systolic dysfunction in 7.3 years (2.12 cases per 100 person-years, $P = 0.093$).

Next, we investigated the prognosis of the 12 subjects who developed systolic dysfunction (Table 3). Systolic dysfunction had a wide range of age for onset (ie, 41 to 74 years; mean, 58.9 ± 11.9 years). Three subjects (25.0%) were 41 to 50 years old, 4 subjects (33.3%) were 51 to 60 years old, 1 subject (8.3 %) had onset age of 62, and 4 subjects (33.3 %) were over 70 years old. Four subjects (33.3%) were male, and the mean age at death or the most recent evaluation was 67.3 ± 11.5 years. All 12 subjects were admitted to hospital for heart failure, and 11 out of 12 subjects (91.7%) died within the follow-up period (8.3 ± 4.0 years). Four out of 11 subjects (36.4%) died of refractory heart failure, 4 subjects (36.4%) died suddenly from causes related to heart failure, 2 subjects (18.2%) died of interstitial pneumonia after heart failure, and 1 subject (9.1%) died of cerebral infarction with atrial fibrillation with use of an appropriate dose of warfarin.

Discussion

In this longitudinal study, 107 of the 157 genotyped HCM subjects underwent serial echocardiography, and during the mean follow-up period of 7 years, 12 subjects out of 107 experienced systolic dysfunction (1.60 cases per 100 person-years). The major finding was, that in a relatively large population of genotyped HCM subjects, EF decreased significantly in the non-*MYBPC3* group (69.0 ± 8.0% to 63.7 ± 13.0%, $P < 0.001$) (Figure 1), and freedom from systolic dysfunction was lower in subjects in the non-*MYBPC3* group than those in *MYBPC3* group (Kaplan-Meier, log-rank test, $P = 0.010$) (Figure 2).

The clinical features of HCM are particularly heterogeneous.[7,8] A patient subset characterized by clinical progression of left ventricular systolic dysfunction has been described.[9–13] Most of the prior literature about systolic dysfunction is, however, limited to isolated or small groups of subjects.[9–13] Recently, Harris et al reported on a cohort of HCM subjects with systolic dysfunction in a multicenter study that included the largest group of subjects with systolic dysfunction reported to date.[23] In that study, incidence of systolic dysfunction was 1.12 cases per 100 person-years. In our study, incidence of systolic dysfunction was 12 cases per 749 person-years of follow-up (1.60 cases per 100 person-years); the rates in these 2 studies were similar, which suggests that the cohort of 157 genotyped HCM subjects in this study was comparable to the cohort of 1259 study subjects in the former study in terms of systolic dysfunction.

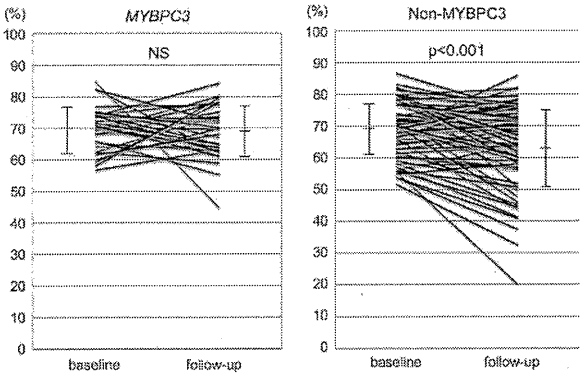

Figure 1. The percent decrease in ejection fraction in *MYBPC3* and non-*MYBPC3* groups. *MYBPC3*, myosin-binding protein C gene; NS, not significant.

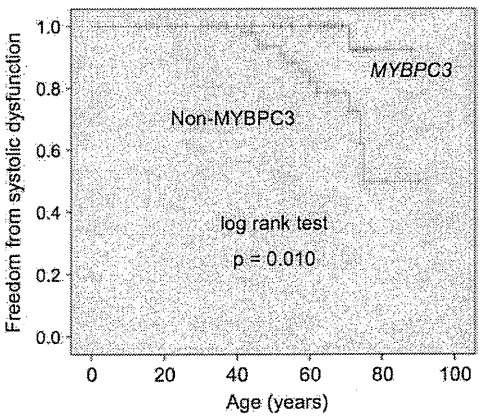

Figure 2. Kaplan-Meier analysis comparing the age at which systolic dysfunction developed in subjects in the myosin-binding protein C (*MYBPC3*) gene and non-*MYBPC3* groups.

Recent advances in molecular genetics have demonstrated that HCM can be considered as a disease of the sarcomere. Some studies have investigated the relationship between specific sarcomere gene mutation and systolic dysfunction; however, these were limited to subjects with mutations in a single gene only.[14–19] In the multicenter cohort of over 1200 HCM subjects,[23] 44 subjects demonstrated systolic dysfunction, and 3 probands were genotyped to HCM-causing sarcomere protein mutations: p.Gly716Arg in *MYH7* in 2 and G-791 in *MYBPC3* in 1. This number of subjects (6.8%, 3 of 44) is very limited. On the other hand, we studied a relatively large population of HCM subjects with several sarcomere gene mutations and analyzed the relationship between disease-causing genes and systolic dysfunction.

We found that freedom from systolic dysfunction was lower in subjects in the non-*MYBPC3* group than those in *MYBPC3* group in this longitudinal study (Kaplan-Meier, log-rank test, $P = 0.010$) (Figure 2). This could be explained by several findings in the recent study. Sakata et al investigated left ventricular function reserve in HCM patients with and without mutations in cardiac troponin genes (*TNNT2* and *TNNI3*) before transition to systolic dysfunction.[28] Interestingly, the group of subjects with troponin gene mutations showed a significant increase in left ventricular end-systolic volume during an exercise test. This suggests that subjects with troponin gene mutations display exercise-induced left ventricular systolic dysfunction more frequently than HCM subjects without troponin gene mutations. The authors speculated that the mechanism of the systolic dysfunction was myocardial ischemia due to lumen narrowing of intramural coronary arteries. It was reported that the use of calcium channel blockers in advance of established clinical disease could prevent HCM caused by sarcomere protein gene mutations in a mouse model,[29] therefore the use of calcium channel blockers to ameliorate ischemia in advance of established systolic dysfunction may be useful to prevent the progression to systolic dysfunction in HCM.

Next, we investigated the prognosis of the 12 subjects who developed systolic dysfunction during follow-up (Table 3). At the time of presentation of systolic dysfunction, the mean age of the 12 subjects was 58.9 years (range, 41–74 years) and 67.3 years (range, 51–84 years) at death or the most recent evaluation. All of the 12 subjects were admitted to the hospital for heart failure, and 11 out of 12 subjects (91.7%) died within the follow-up period, which means the prognosis of genotyped subjects with HCM demonstrating systolic dysfunction is poor. The present study demonstrated that HCM subjects with mutations in sarcomere-related genes other than *MYBPC3* develop left ventricular systolic dysfunction more frequently than those with *MYBPC3* mutations, and most of those who developed systolic dysfunction died within mean period of 8.3 years. These observations may provide important prognostic information in the clinical practice of HCM, which suggests that subjects with mutations in sarcomeric genes require careful management for systolic dysfunction from the point of view of prognosis.

There remain several limitations of the present study. First, in recent studies, multiple gene mutations have been reported, which may further contribute to the disease heterogeneity.[30–35] We did not include carriers with multiple mutations in this study to compare the differences in the clinical course between multiple disease-causing genes; however, we should also assess the influence of multiple mutations on HCM phenotype as the next step. Second, heart transplantation should have been considered for many subjects who showed systolic dysfunction in our study; however, heart transplantation is not common in Japan. It

Table 3. Clinical Data From 12 Subjects Who Showed Systolic Dysfunction During Follow-up

No.	Gene	Gender	A, y	Last Evaluation	B, y	A to B, y	Etiology of Death
1	*MYBPC3*	Male	71	Death	76	5	Interstitial pneumonia after heart failure
2	*MYH7*	Male	74	Death	78	4	Sudden death during heart failure
3	*TNNT2*	Female	60	Death	69	9	Refractory heart failure
4	*TNNT2*	Female	53	Death	62	9	Refractory heart failure
5	*TNNI3*	Female	46	Death	52	6	Sudden death during chronic heart failure
6	*TNNI3*	Female	52	Death	59	7	Sudden death during heart failure
7	*TNNI3*	Female	45	Death	51	6	Refractory heart failure
8	*TNNI3*	Female	57	Death	74	17	Cerebral infarction with atrial fibrillation
9	*TNNI3*	Female	71	Death	84	13	Refractory heart failure
10	*TNNI3*	Male	62	Death	70	8	Interstitial pneumonia after hear failure
11	*TNNI3*	Male	75	Death	79	4	Sudden death during chronic heart failure
12	*TNNI3*	Female	41	Alive	53	12	Not available
			58.9 ± 11.9		67.3 ± 11.5	8.3 ± 4.0	

Abbreviations: A, age at systolic dysfunction; A to B, interval from A to B; B, age at death or last evaluation.

Published online in Wiley Online Library (wileyonlinelibrary.com)
DOI:10.1002/clc.22082

appears that without the option of heart transplantation, the natural course of systolic dysfunction related to sarcomere genes mutations often follows an adverse course with fatal outcome. Last, 46 out of the 71 subjects in the non-*MYBPC3* group were *TNNI3* mutation carriers (Table 2), which might reflect a founder effect. However, we also included subjects with mutations in *MYBPC3* that were reported to be associated with systolic dysfunction.[18,19]

Conclusion

Non-*MYBPC3* mutation carriers developed left ventricular systolic dysfunction more frequently than *MYBPC3* mutation carriers, and the majority of sarcomere gene mutation carriers with systolic dysfunction had fatal outcomes during follow-up. This suggests that subjects with mutations in sarcomeric genes require careful management for systolic dysfunction.

References

1. Seidman JG, Seidman C. The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms. *Cell.* 2001;104:557–567.
2. Maron BJ, Shirani J, Poliac LC, et al. Sudden death in young competitive athletes. Clinical, demographic, and pathological profiles. *JAMA.* 1996;276:199–204.
3. Maron BJ, Gardin JM, Flack JM, et al. Prevalence of hypertrophic cardiomyopathy in a general population of young adults. Echocardiographic analysis of 4111 subjects in the CARDIA Study. Coronary Artery Risk Development in (Young) Adults. *Circulation.* 1995;92:785–789.
4. Arad M, Seidman JG, Seidman CE. Phenotypic diversity in hypertrophic cardiomyopathy. *Hum Mol Genet.* 2002;11:2499–2506.
5. Kimura A, Harada H, Park JE, et al. Mutations in the cardiac troponin I gene associated with hypertrophic cardiomyopathy. *Nat Genet.* 1997;16:379–382.
6. Spirito P, Seidman CE, McKenna WJ, et al. The management of hypertrophic cardiomyopathy. *N Engl J Med.* 1997;336:775–785.
7. Maron BJ. Hypertrophic cardiomyopathy: a systematic review. *JAMA.* 2002;287:1308–1320.
8. Maron BJ, McKenna WJ, Danielson GK, et al. American College of Cardiology/European Society of Cardiology clinical expert consensus document on hypertrophic cardiomyopathy. *J Am Coll Cardiol.* 2003;42:1687–1713.
9. Maron BJ, Spirito P. Implications of left ventricular remodeling in hypertrophic cardiomyopathy. *Am J Cardiol.* 1998;81:1339–1344.
10. Spirito P, Maron BJ, Bonow RO, et al. Occurrence and significance of progressive left ventricular wall thinning and relative cavity dilatation in patients with hypertrophic cardiomyopathy. *Am J Cardiol.* 1987;60:123–129.
11. Maron BJ, Epstein SE, Roberts WC. Hypertrophic cardiomyopathy and transmural myocardial infarction without significant atherosclerosis of the extramural coronary arteries. *Am J Cardiol.* 1979;43:1086–1102.
12. Shirani J, Maron BJ, Cannon RO, et al. Clinicopathologic features of hypertrophic cardiomyopathy managed by cardiac transplantation. *Am J Cardiol.* 1993;72:434–440.
13. Hecht GM, Klues HG, Roberts WC, et al. Coexistence of sudden cardiac death and end-stage heart failure in familial hypertrophic cardiomyopathy. *J Am Coll Cardiol.* 1993;22:489–497.
14. Kokado H, Shimizu M, Yoshio H, et al. Clinical features of hypertrophic cardiomyopathy caused by a Lys183 deletion mutation in the cardiac troponin I gene. *Circulation.* 2000;102:663–669.
15. Fujino N, Shimizu M, Ino H, et al. Cardiac troponin T Arg92Trp mutation and progression from hypertrophic to dilated cardiomyopathy. *Clin Cardiol.* 2001;24:397–402.
16. Fujino N, Shimizu M, Ino H, et al. A novel mutation Lys273Glu in the cardiac troponin T gene shows high degree of penetrance and transition from hypertrophic to dilated cardiomyopathy. *Am J Cardiol.* 2002;89:29–33.
17. Shimizu M, Ino H, Okeie K, et al. Septal wall thinning and systolic dysfunction in patients with hypertrophic cardiomyopathy caused by a cardiac troponin I gene mutation. *Am Heart J.* 2002;143:690–695.
18. Konno T, Shimizu M, Ino H, et al. A novel missense mutation in the myosin binding protein-C gene is responsible for hypertrophic cardiomyopathy with left ventricular dysfunction and dilation in elderly patients. *J Am Coll Cardiol.* 2003;41:781–786.
19. Kubo T, Kitaoka H, Okawa M, et al. Lifelong left ventricular remodeling of hypertrophic cardiomyopathy caused by a founder frameshift deletion mutation in the cardiac myosin-binding protein C gene among Japanese. *J Am Coll Cardiol.* 2005;46:1737–1743.
20. Niimura H, Bachinski LL, Sangwatanaroj S, et al. Mutations in the gene for cardiac myosin-binding protein C and late-onset familial hypertrophic cardiomyopathy. *N Engl J Med.* 1998;338:1248–1257.
21. Richardson P, McKenna W, Bristow M, et al. Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of cardiomyopathies. *Circulation.* 1996;93:841–842.
22. Chiu C, Bagnall RD, Ingles J, et al. Mutations in Alpha-Actinin-2 Cause Hypertrophic Cardiomyopathy. *J Am Coll Cardiol.* 2010;55:1127–1135.
23. Harris KM, Spirito P, Maron MS, et al. Prevalence, clinical profile, and significance of left ventricular remodeling in the end-stage phase of hypertrophic cardiomyopathy. *Circulation.* 2006;114:216–225.
24. Konno T, Shimizu M, Ino H, et al. A novel mutation in the cardiac myosin-binding protein C gene is responsible for hypertrophic cardiomyopathy with severe ventricular hypertrophy and sudden death. *Cli Sci (Lond).* 2006;110:125–131.
25. Uchiyama K, Hayashi K, Fujino N, et al. Impact of QT Variables on clinical outcome of genotyped hypertrophic cardiomyopathy. *Ann Noninvasive Electrocardiol.* 2009;14:65–71.
26. Funada A, Konno T, Fujino N, et al. Impact of renin-angiotensin system polymorphisms on development of systolic dysfunction in hypertrophic cardiomyopathy. Evidence from a study of genotyped patients. *Circ J.* 2010;74:2674–2680.
27. CardioGenomics. http://cardiogenomics.med.harvard.edu/home. Accessed January 24, 2012.
28. Sakata K, Ino H, Fujino N, et al. Exercise-induced systolic dysfunction in patients with non-obstructive hypertrophic cardiomyopathy and mutations in the cardiac troponin genes. *Heart.* 2008;94:1281–1287.
29. Semsarian C, Ahmad I, Giewat M, et al. The L-type calcium channel inhibitor diltiazem prevents cardiomyopathy in a mouse model. *J Clin Invest.* 2002;109:1013–1020.
30. Ho CY, Lever HM, DeSanctis R, et al. Homozygous mutation in cardiac troponin T implications for hypertrophic cardiomyopathy. *Circulation.* 2000;102:1950–1955.
31. Richard P, Charron P, Leclercq C, et al. Homozygotes for a R869G mutation in the beta -myosin heavy chain gene have a severe form of familial hypertrophic cardiomyopathy. *J Mol Cell Cardiol. 2000;*32:1575–1583.
32. Richard P, Charron P, Carrier L, et al. EUROGENE Heart Failure Project. Hypertrophic cardiomyopathy: distribution of disease genes, spectrum of mutations, and implications for a molecular diagnosis strategy. *Circulation.* 2003;107:2227–2232.
33. Van Driest SL, Vasile VC, Ommen SR, et al. Myosin binding protein C mutations and compound heterozygosity in hypertrophic cardiomyopathy. *J Am Coll Cardiol.* 2004;44:1903–1910.
34. Ingles J, Doolan A, Chiu C, et al. Compound and double mutations in patients with hypertrophic cardiomyopathy: implications for genetic testing and counselling. *J Med Genet. 2005;*42:e59.
35. Kubo T, Kitaoka H, Okawa M, et al. Genetic screening and double mutation in Japanese patients with hypertrophic cardiomyopathy. *Circ J.* 2011;75:2654–2659.

Published online in Wiley Online Library (wileyonlinelibrary.com)
DOI:10.1002/clc.22082

RESEARCH ARTICLE

Human Mutation

OFFICIAL JOURNAL
HGVS
HUMAN GENOME VARIATION SOCIETY
www.hgvs.org

CDH13 Gene Coding T-Cadherin Influences Variations in Plasma Adiponectin Levels in the Japanese Population

Hiroko Morisaki,[1] Itaru Yamanaka,[1] Naoharu Iwai,[2] Yoshihiro Miyamoto,[3,4] Yoshihiro Kokubo,[4] Tomonori Okamura,[4] Akira Okayama,[4] and Takayuki Morisaki[1,5]*

[1]*Departments of Bioscience and Genetics;* [2]*Genomic Medicine, National Cerebral and Cardiovascular Center Research Institute, Osaka, Japan;* [3]*Departments of Atherosclerosis, Metabolism and Clinical Nutrition;* [4]*Preventive Cardiology, National Cerebral and Cardiovascular Center, Osaka, Japan;* [5]*Department of Molecular Pathophysiology, Osaka University Graduate School of Pharmaceutical Sciences, Osaka, Japan*

Communicated by Michael Dean

Received 13 May 2011; accepted revised manuscript 24 October 2011.
Published online 7 November 2011 in Wiley Online Library (www.wiley.com/humanmutation).DOI: 10.1002/humu.21652

ABSTRACT: Adiponectin is most abundantly expressed in adipose tissue and well known to play an important role in metabolic regulation. Several studies have attempted to identify the genetic determinants of metabolic syndrome (MetS), though no study has revealed a *cis*- or *trans*-single nucleotide polymorphism (SNP) that affects plasma adiponectin levels, except the adiponectin structure gene and genes encoding adiponectin-regulatory proteins. We performed a genome-wide association study in regards to plasma adiponectin concentrations in 3,310 Japanese subjects. We identified the strongest statistically associated SNP (rs4783244) with adiponectin levels ($P = 3.8 \times 10^{-19}$) in the first intron of *CDH13* (T-cadherin) gene in a 30-kb haplotype block covering the promoter region to first intron. In addition, rs12051272 SNP genotypes in linkage disequilibrium with rs4783244 were found to be more significantly associated with adiponectin levels ($P = 9.5 \times 10^{-20}$) and specifically with the levels of high-molecular weight (HMW) adiponectin, a subtype form associated with parameters related to glucose metabolism. Our results did show more significant association with adiponectin levels than rs12444338 (in CDH13) SNP genotypes reported recently. We suggest that the phenotype-affecting haplotype tagged by rs12051272 SNP would affect the plasma adiponectin levels and that we have to take the CDH13 genotype into account before considering the functional relevance of the adiponectin level.

Hum Mutat 33:402–410, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: GWAS; CDH13; adiponectin; SNP

Introduction

Adiponectin is one of the most abundant gene products expressed in adipose tissue [Maeda et al., 1996]. It is also well known to play an important role in metabolic regulation affecting obesity, insulin sensitivity, or atherosclerosis [Yamauchi et al., 2001]. Plasma adiponectin levels are known to be correlated with body mass index (BMI), type 2 diabetes mellitus (T2DM), or even coronary artery disease [Hotta et al., 2000]. Several studies have also shown that adiponectin plays many metabolic effects including antidiabetic, antiatherosclerotic, or anti-inflammatory action [Matsuzawa et al., 2004]. Also, it is suggested to affect the relationship between obesity and insulin resistance or T2DM [Kern et al., 2003]. Production of adiponectin and plasma adiponectin concentrations was known to be regulated by complexed mechanisms [Yu and Ginsberg, 2005]. For example, its expression is increased by leanness, adrenalectomy, insulin-like growth factor 1, ionomycin, or thiazolidinediones, while it is decreased by obesity, tumor necrosis factor-α, glucocorticoids, β-adrenergic agonists, or cyclic AMP. In addition, genetic factors are also suggested to regulate adiponectin concentration as shown by the family study [Menzaghi et al., 2007] or several genome-wide linkage scans [Yang and Chuang, 2006]. Several candidate genes include the adiponectin structural gene (*ADIPOQ*; MIM# 605441) as well as the genes encoding adiponectin–regulatory proteins have been postulated to influence the adiponectin concentration [Ntalla et al., 2009], though the role of genetic variants regulating adiponectin function on insulin resistance, T2DM, or coronary artery disease, has not been clearly determined.

Recently, the development of low-cost, high-throughput genotyping technology made it possible to identify common genetic variants influencing health outcomes on a genome-wide scale. Several studies were performed to identify the genetic determinants of metabolic syndrome (MetS) and related traits. In one study, a comprehensive assessment of the genetic determinants of adiponectin levels was performed on a genome-wide basis in northern and western European population in addition to genome-wide linkage and association analyses, and the genetic influences on plasma levels of adiponectin were evaluated [Ling et al., 2009].

Here, to identify genes influencing variation in plasma adiponectin levels, we performed a genome-wide association study on plasma adiponectin concentration in subjects recruited in Suita, Osaka, Japan.

Materials and Methods

Suita Study

The Suita study was initiated as a cohort study for cardiovascular diseases of urban residents of Japanese in 1989. The details of this study were described elsewhere [Iwai et al., 2002]. Data from 5,098

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Takayuki Morisaki, National Cerebral and Cardiovascular Center Research Institute, Bioscience and Genetics, 5-7-1 Fujishirodai, Suita, Osaka, 565-8565, Japan. E-mail: morisaki@ri.ncvc.go.jp

participants (2,404 men and 2,694 women) were initially included in the analysis. DNA samples were prepared from 3,310 participants (1,527 men and 1,783 women) after informed consent was obtained. This cohort study was approved by the ethics committee of the National Cardiovascular Center. Subjects received a physical exam during which height, weight, and waist circumference were measured according to a standardized protocol. Blood samples were collected after a 12-hr fast. Plasma adiponectin level and other blood chemical levels including serum total cholesterol (TC), high density lipoprotein (HDL) cholesterol, triglyceride (TG), glucose, and insulin were measured. Hypertension was defined as either a systolic blood pressure (SBP) ≥140 mm Hg, a diastolic blood pressure (DBP) ≥90 mm Hg, or the use of antihypertensive agents. Diabetes was defined as a fasting serum glucose ≥7.0 mmol/L (126 mg/dl), the use of antidiabetic agents, or both. MetS was defined using modified NCEP-ATP III criteria [Heng et al., 2006] based on the International Obesity Task Force central obesity criteria for Asia [Kanazawa et al., 2002].

Yahaba Study

The Yahaba study was initiated as a cohort study for cardiovascular diseases of rural residents in 2007. DNA samples were prepared from 172 participants (69 men and 103 women) after informed consent was obtained. This cohort study was also approved by the ethics committee of the National Cardiovascular Center. Blood samples were collected as the Suita study. The samples were also used for the measurement of the same parameters performed in the Suita study.

Adiponectin Measurement

Plasma adiponectin level was measured using an enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden). Prior to genetic analyses, mean levels of adiponectin and other traits were compared between groups using linear regression in SAS version 9 (SAS Institute, Cary, NC).

Association Study

The association study was initially carried out to identify specific single nucleotide polymorphisms (SNPs) associated with variation in log-transformed adiponectin levels in the Suita Study. Genotyping was initially performed using the Illumina 550K chip (Illumina, San Diego, CA). TaqMan system (Life Technologies, Carlsbad, CA) was then used for further genotyping of the individual SNPs. Exclusion criteria of SNP genotypes for this study were minor allele frequency (MAF) <0.1, *P*(Hardy–Weinberg equilibrium) <0.05, or typing rate <0.98. The genome-wide SNP association analysis for adiponectin was performed in a simple linear regression and additive genetic model without adjustment. In addition to reporting marker-wise statistical test results, genome-wide levels of statistical significance were calculated by applying a Bonferroni correction. Linkage disequilibrium structure was evaluated at selected locus regions using the Haploview software (Broad Institute, Cambridge, MA). For further genotyped individual SNPs, association analysis was performed in a multiple linear regression model with adjustment for age, sex, and BMI.

Results

The statistical analysis in the initial stage (Suita-1) was performed with the data from 842 individuals of Suita study. Based on the exclusion criteria of SNP genotypes, 348,622 SNPs were analyzed. Figure 1 shows genome-wide association with plasma adiponectin as well as a plot of the *P*-values of each SNP according to its physical location encompassing *CDH13* gene (MIM# 601364) region in the initial screening of samples of Suita study. In this analysis, genomic inflation factor (based on median chi-squared) was 1 and cutoff *P*-value after applying a Bonferroni correction was 1.43×10^{-7}. Seven SNPs (four SNPs in choromosome 11 and three SNPs in chromosome 16) were significantly associated with plasma adiponectin. Since these SNPs in chromosome 16 (rs4783244:G>T, rs9940180:C>T, rs7193788:A>G) were located in *CDH13* gene but SNPs in chromosome 11 (rs563272:C>T at 115512749, rs483058:A>G at 115513725, rs7125373:A>G at 115532483, rs1621764:C>T at 115538977) were not located within or near the known genes, we concentrated SNPs located in chromosome 16 in this study. We also evaluated the linkage disequilibrium plots of these SNPs in chromosome 16. The strongest statistically associated SNP (rs4783244) lay in the first intron of *CDH13* gene in a 30-kb haplotype block covering the promoter region to first intron. Haplotype analysis using seven SNPs in this block (rs16957844:A>C, rs3844412:C>T, rs3865186:C>T, rs9940180, rs7193788, rs4783244, rs8047711:A>G) revealed that the haplotype with the strong evidence of association was indeed tagged by a single tag SNP, rs4783244 (Supp. Table S1). Since the selection of tag SNPs in Illumina Infinium II assay was based on the haplotype data of Caucasian population, we next evaluated the haplotype block structure in Japanese population using genotype data available from the international HapMap project. Although the extent of haplotype block of this region in Japanese was similar with that in Caucasians based on the calculated linkage disequilibrium (LD) measure r^2 (Supp. Fig. S1), major haplotype structure consisting the haplotype block including rs4783244 was quite different, suggesting we should reassess the tag SNP selection using Japanese set of SNP data. All HapMap SNPs available in 33-kb region bounded by SNPs rs2318177:C>T and rs8045889:C>T that covers the 30-kb haplotype block, were screened by LD measure—presenting the $r^2 < 0.7$ with all tag SNPs in Illumina assay system. Seven SNPs (rs11646213:A>T, rs12051272:G>T, rs16957848:C>T, rs3852729:A>C, rs3865183:A>G, rs6565051:A>G, rs8060461:A>G) met these criteria. Of these, six SNPs (rs11646213, rs16957848, rs3852729, rs3865183, rs6565051, rs8060461) were excluded since they showed stronger r^2 with other SNPs presented in Illumina assay system than with rs4783244 (Supp. Table S2). So, the TaqMan probe set predesigned for the remained rs12051272, which had been excluded from Illumina assay system because of a low MAF in Caucasian population (Supp. Table S2), was obtained and genotyped in the same Japanese population. Interestingly, the rs12051272 SNP presented even stronger association with plasma adiponectin levels than rs4783244 ($P = 2.6 \times 10^{-13}$ for unadjusted, and 9.5×10^{-20} adjusted for sex, age, and BMI) in this initial stage study (Suita-1).

Next we genotyped these two SNPs, rs4783244 and rs12051272, in the whole set of Suita Study subjects using a TaqMan PCR method (Table 1). We intensively included the samples from the first screening to measure the typing discrepancy between two methods. The replicate error rate between two methods in rs4783244 was 0.59%. Those five subjects with ambiguous data were accordingly excluded from the further analysis.

The statistical analysis in the second stage (Suita-2) was then conducted with the data from remaining 2,468 Suita study participants left out from the first screening (Table 1). The basic characteristics of these two groups are similar as shown in Table 2. The mean age was older in the second group as expected since the aged participants were excluded from the first screening. Accordingly, the mean adiponectin level, which was known to be affected by age, was higher

HUMAN MUTATION, Vol. 33, No. 2, 402–410, 2012

Table 1. Results of Genotyping Two SNPs, rs4783244 and rs12051272, in the *CDH13* Gene, in Suita-1, Suita-2, or Yahaba

Set				Suita-1					Suita-2					Yahaba				
Number(M/F)				373/464					1144/1311					68/101				
						P-value					*P*-value					*P*-value		
SNP	MAF			Mean	SD	Unadj	a,s adj	a,s,B adj	Mean	SD	Unadj	a,s adj	a,s,B adj	Mean	SD	Unadj	a,s adj	a,s,B adj
rs4783244	0.31	Female	11	6.26	4.96	3.56×10^{-8}	1.51×10^{-6}	8.98×10^{-9}	8.00	5.57	2.26×10^{-9}	2.09×10^{-8}	2.35×10^{-10}	9.53	4.60	0.32	0.33	0.30
			12	8.06	5.42				8.60	5.71				11.4	6.95			
			22	9.78	6.49				10.0	6.38				11.8	8.82			
		Male	11	3.60	1.57	5.17×10^{-9}	1.63×10^{-9}	1.11×10^{-12}	4.72	3.62	8.14×10^{-10}	4.85×10^{-11}	6.30×10^{-13}	4.91	5.22	5.88×10^{-5}	6.33×10^{-5}	7.32×10^{-5}
			12	5.12	3.23				5.79	4.54				6.03	5.29			
			22	6.12	4.34				6.64	4.98				10.0	4.58			
		Combined	11	4.80	3.60	7.87×10^{-13}	1.55×10^{-16}	3.83×10^{-19}	6.30	5.25	4.24×10^{-15}	2.31×10^{-17}	1.60×10^{-21}	7.31	6.33	0.0031	0.0007	0.0006
			12	6.65	4.89				7.15	5.58				9.06	7.69			
			22	7.87	6.06				8.26	6.25				11.0	6.96			
rs12051272	0.31	Female	11	5.83	4.28	3.82×10^{-9}	1.63×10^{-9}	1.45×10^{-9}	7.94	5.55	1.53×10^{-9}	1.18×10^{-8}	1.49×10^{-10}	9.53	4.60	0.45	0.45	0.42
			12	8.12	5.40				8.58	5.78				11.6	6.92			
			22	9.70	6.60				10.0	6.33				11.6	8.78			
		Male	11	3.65	1.65	8.81×10^{-9}	3.22×10^{-9}	2.30×10^{-12}	4.68	3.62	7.60×10^{-10}	4.39×10^{-11}	5.32×10^{-13}	4.91	5.22	5.88×10^{-5}	6.33×10^{-5}	7.32×10^{-5}
			12	5.08	3.23				5.78	4.53				6.03	5.29			
			22	6.11	4.31				6.63	4.95				10.0	4.58			
		Combined	11	4.65	3.23	2.61×10^{-13}	2.82×10^{-17}	9.46×10^{-20}	6.25	5.24	2.61×10^{-15}	1.03×10^{-17}	8.07×10^{-22}	7.31	6.33	0.0047	0.0013	0.0011
			12	6.68	4.93				7.14	5.61				9.12	7.77			
			22	7.85	6.08				8.25	6.21				10.9	6.96			

M, male; F, female; MAF, minor allele frequency; 1, minor allele; 2, major allele; unadj, unadjusted; a,s adj, age and sex adjusted; a,s,B adj, age, sex, and BMI adjusted.

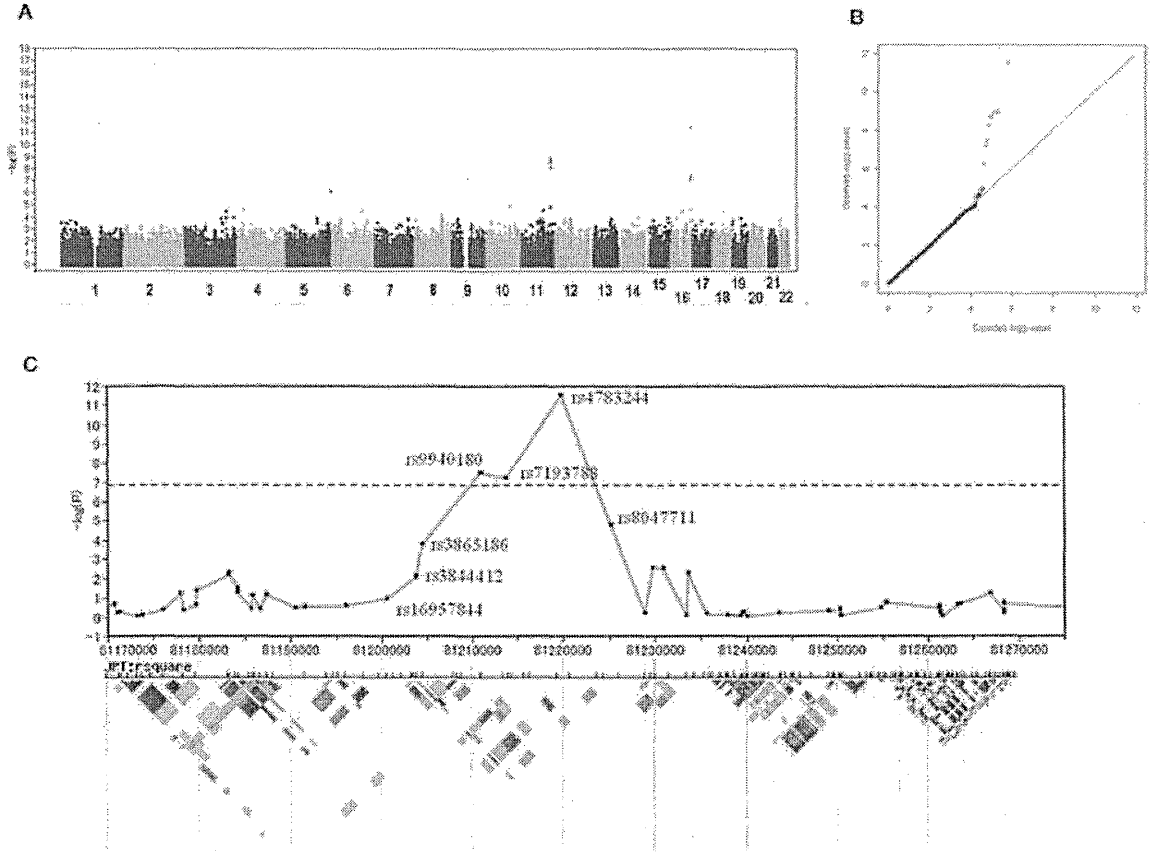

Figure 1. Genome-wide association with plasma adiponectin in Suita population. **A:** Manhattan plot. **B:** PP plot. **C:** *P*-values of each SNPs according to its physical location encompassing *CDH13* gene region were shown.

in the second group ($P < 0.001$). But the intergroup difference in plasma adiponectin levels was not significant after adjustment for age and sex ($P = 0.23$). Using the second group, the two SNPs examined showed again statistically significant association with log-transformed plasma adiponectin levels ($P = 4.2 \times 10^{-15}$ and 2.6×10^{-15} for rs4783244 and rs12051272, respectively). The association was even more significant after adjustment for sex, age, and BMI ($P = 1.6 \times 10^{-21}$ and 8.1×10^{-22}). The association of rs12051272 alleles was slightly more significant than that of rs4783244, same as the result for the first group ($P = 3.8 \times 10^{-19}$ and 9.5×10^{-20}, respectively after adjustment for sex, age, and BMI). The evidence of association was strongest ($P = 4.4 \times 10^{-37}$ and 6.9×10^{-38}) when data from two groups were combined (Suita-1 + Suita-2) and adjusted for sex, age, and BMI (Supp. Table S3). Finally, pairwise LD (r^2) between rs4783244 and rs12051272 in Suita study group was 0.97. From these data, we concluded that the haplotype tagged by rs12051272 was most responsible for the association with plasma adiponectin levels.

Table 2. The Basic Characteristics of Groups for the Study

	Suita-1	Suita-2	Yahaba	
Number	842	2468	172	
Female sex percent	55.6	53.3	59.9	
Age	58.2 (7.0)	66.7 (11.3)	62.2 (6.6)	b,c
BMI (kg/m^2)	22.8 (2.9)	22.9 (3.2)	24.3 (3.1)	c
Waist/hip ratio	0.884 (0.046)	0.907 (0.052)	0.913 (0.073)	b,c
Adiponectin (μg/dl)	7.0 (5.5)	7.5 (5.9)	9.8 (7.6)	b,c
TC (mg/dl)[a]	211.5 (32.5)	207.4 (32.3)	211.0 (32.8)	b
LDLc (mg/dl)	128.4 (31.1)	126.1 (29.5)	131.6 (32.0)	
HDLc (mg/dl)	61.5 (15.6)	59.9 (15.6)	60.3 (14.0)	b
TG (mg/dl)	92.7 (65.7)	93.4 (61.6)	100.9 (58.9)	c
SBP (mm Hg)[a]	123.1 (17.3)	131.4 (19.6)	122.8 (20.1)	b,c
DBP (mm Hg)	77.4 (10.0)	78.0 (10.0)	73.0 (12.0)	c
Fasting insulin (μU/ml)	4.4 (3.3)	4.8 (3.8)	5.5 (4.9)	b,c
Fasting glucose (mg/dl)[a]	94.8 (14.4)	99.2 (18.8)	88.1 (14.2)	b,c

All measures were shown in mean (SD).
[a]Variables were log tranformed for initial calculation of mean and SD. Values in table were shown as untransformed values.
[b]Statistically significant difference.
[c]Statistically significant difference between Suita (1 + 2) and Yahaba cohort.

Since the first and second groups were derived from the single cohort, though they did not overlap each other, we next conducted an additional analysis using the data from Yahaba study, another residential cohort study originated in northern part of Japan to confirm our results from Suita study. The population characteristics of these two studies were different in several points. The cohort in Suita study are residents in the urban area of Osaka, the second largest city of Japan, and most of the residents originated in western part of Japan, and settled down about half a century ago. In contrast, Yahaba is a small town located in the rural area of northern part of Japan and its

Table 3. SNP Genotypes in CDH13 and Adiponectin Types in Yahaba

				HMW adiponectin					Non-HMW adiponectin				
				Additive *P*-value					Additive *P*-value				
SNP	MAF		Genotype	Mean	SD	Unadj	a,s adj	a,s,B adj	Mean	SD	Unadj	a,s adj	a,s,B adj
rs4783244	0.30	Female	22	7.43	6.96	0.46	0.46	0.42	4.67	2.19	0.15	0.15	0.13
			12	7.36	5.15				4.26	1.84			
			11	6.00	3.15				3.72	1.45			
		Male	22	6.38	3.48	4.10×10^{-5}	4.50×10^{-5}	3.80×10^{-5}	3.69	1.38	0.24	0.23	0.38
			12	3.04	3.39				3.31	1.77			
			11	3.33	2.36				3.05	1.10			
		Combined	22	6.95	5.40	0.0071	0.001	0.001	4.24	1.93	0.113	0.062	0.056
			12	5.35	5.94				3.92	1.86			
			11	4.86	3.42				3.48	1.34			
rs12051272	0.30	Female	22	7.33	6.86	0.56	0.56	0.52	4.61	2.21	0.22	0.22	0.20
			12	7.46	5.19				4.32	1.82			
			11	6.00	3.15				3.72	1.45			
		Male	22	6.38	3.48	4.13×10^{-5}	4.50×10^{-5}	3.83×10^{-5}	3.69	1.38	0.24	0.23	0.38
			12	3.04	3.39				3.31	1.77			
			11	3.33	2.36				3.05	1.10			
		Combined	22	6.91	5.36	0.009	0.002	0.002	4.21	1.94	0.156	0.095	0.089
			12	5.37	6.02				3.95	1.85			
			11	4.86	3.42				3.48	1.34			

MAF, minor allele frequency; 1, minor allele; 2, major allele; unadj, unadjusted; a,sadj, age and sex adjusted; a,s,B adj, age, sex, and BMI adjusted.

residents were mostly indigenous since 1800s. The rs4783244 and rs12051272 SNPs were genotyped using the TaqMan PCR method as described earlier. Although the sample size in Yahaba was smaller compared to the previous two studies, we found a statistically significant association ($P = 0.0006$ and 0.0011, respectively) after male and female were combined. Here, female samples in Yahaba did not show significant association between adiponectin levels and either SNP genotype under any genetic model including a dominant model (data not shown), while the MAF in Yahaba was 0.31 and not different from that for Suita study. This might be due to small number of Yahaba study or characteristics of Yahaba female. *CDH13* gene codes for T-cadherin that had been identified as a receptor specific for high-molecular weight (HMW) adiponectin [Hug et al., 2004]. We next examined if the association was specific for the levels of HMW adiponectin and not for that of low-molecular weight (LMW) adiponectin using data from Yahaba study. As expected, rs12051272 SNP genotypes were significantly associated only with the levels of HMW adiponectin ($P = 0.0018$ after adjustment for sex, age, and BMI) and not with that of other types of adiponectin ($P = 0.09$) (Table 3). However, there was weaker association between SNP genotype and HMW adiponectin in female than in male partly because of sex difference in plasma adiponectin concentrations (female: 11.4 μg/dl, male: 7.7 μg/dl, $P = 9 \times 10^{-6}$). Since several SNPs or mutant within the *ADIPOQ* gene, which codes for adiponectin, had been reported to be associated with plasma adiponectin levels previously, we genotyped these SNPs additionally. These included rs2241766:G>T (Gly15:exon1), rs1501299:A>C (intron1), rs710445:A>G (promoter), and Ile164Thr (nonsynonymous substitution). They indeed showed a weak but statistically significant association with adiponectin levels in our Suita study subjects ($P = 0.00093$, 0.32, 0.0001, 2.2×10^{-25}, respectively), but the magnitude of significance was smaller compared that of *CDH13* SNP rs12051272. The effect size presented in eta-squared for rs12051272, rs2241766, rs1501299, rs710445, and Ile164Thr was 3.28%, 0.27%, 0.02%, 0.37%, and 2.62%, respectively. The beta-coefficient per allele for them was 27.7%, 8.0%, 2.4%, 8.7%, and 127.5%, respectively. Ile164Thr mutant showed the largest beta-coefficient, but the MAF was very low (0.8%), resulting in smaller eta value than that of rs12051272 (Table 4).

Since T-cadherin has been shown to bind low density lipoprotein (LDL) cholesterol [Resink et al., 1999], we next analyzed the association between rs12051272 genotypes and the levels of LDL cholesterol using the data from whole Suita study participants. The rs12051272 genotypes showed no significant association with serum LDL cholesterol levels ($P = 0.19$), as well as with total or HDL cholesterol levels ($P = 0.46$ and 0.16, respectively). Plasma adiponectin levels also have been shown independently related with many obesity-related phenotypes, including insulin resistance [Lindsay et al., 2002; Snehalatha et al., 2003], the levels of fasting insulin, fasting glucose, and fasting TG. Since they showed a significant association also in our study, we next evaluated the association between those phenotypes and the rs12051272 genotypes (Table 5A). Interestingly, although we failed to find any statistical association between them in simple regression analysis adjusted for age and sex, we found a significant association when log-transformed plasma adiponectin level was included as an explanatory variable. The partial correlation coefficient between plasma adiponectin levels and homeostasis model assessment-estimated insulin resistance (HOMA-IR), the levels of fasting insulin, fasting glucose, and fasting TG in Suita study population was −0.39 ($P = 5.00 \times 10^{-102}$), −0.38 ($P = 7.04 \times 10^{-94}$), −0.23 ($P = 1.77 \times 10^{-34}$), and −0.36 ($P = 4.48 \times 10^{-85}$), respectively, while that of plasma adiponectin levels and rs12051272 genotypes (additive model) was −0.24 ($P = 2.0 \times 10^{-34}$). Similarly, that of

Table 4. Effect Size of SNPs on Serum Adiponectin Levels

		Serum adiponectin (μg/ml), mean (SD)				Eta squared			Beta coefficient		
	Allele1 f	Allele 1	Both	Allele2	*P* value (a)	SNP (a)	Age	Sex	SNP (*p* al)	Age	Sex (*p* sex)
rs12051272	0.688	8.01 (0.10)	6.87 (0.09)	5.75 (0.17)	2.0×10^{-31}	3.28%	5.77%	15.35%	27.7%	24.2%	79.1%
rs4783244	0.684	8.03 (0.11)	6.88 (0.09)	5.84 (0.17)	1.7×10^{-30}	3.17%	5.71%	15.43%	27.1%	24.1%	79.3%
rs2241766	0.295	7.66 (0.24)	7.45 (0.11)	7.05 (0.09)	9.3×10^{-1}	0.27%	5.82%	15.30%	8.0%	24.3%	79.0%
rs1501299	0.277	7.18 (0.23)	7.2 (0.11)	7.33 (0.09)	0.32	0.02%	5.93%	15.14%	2.4%	24.5%	78.6%
rs266729	0.748	7.4 (0.09)	7.16 (0.11)	6.77 (0.24)	0.009	0.17%	5.84%	15.29%	6.6%	24.3%	79.0%
rs710445	0.612	7.72 (0.17)	7.33 (0.10)	6.97 (0.11)	1.6×10^{-4}	0.37%	5.84%	15.14%	8.7%	24.3%	78.6%
rs822395	0.063	5.02 (0.86)	7.01 (0.19)	7.31 (0.07)	0.029	0.12%	5.84%	15.23%	10.0%	24.3%	78.8%
APM1(I164T)	0.008	NA	3.5 (0.25)	7.35 (0.07)	2.2×10^{-25}	2.62%	5.95%	15.51%	127.5%	24.6%	79.5%
APM1(H241P)	0.006	NA	8.44 (0.71)	7.25 (0.07)	0.062	0.09%	5.89%	15.25%	26.1%	24.4%	78.8%

Age, sex, and BMI adjusted ($N = 3310$).
f, frequency; (a), additive; (p al), per allele; (p sex), per sex.

Table 5. Association of Obesity-Related Phenotypes, Adiponectin, and the rs12051272 Genotypes in Suita (1 + 2)

	P-value (response variable = each parameter)				Partial correlation coefficient		
	rs12 (a v)	rs12 (a v) pl adipo adj	pl adipo	pl adipo, rs12 (a v) adj	betw rs12 (a v) and each	betw rs12 (a v) and pl adipo	betw pl adipo and each
			A. Adjustment of age and sex				
Fasting insulin[a]	0.84	4.05×10^{-6}	9.09×10^{-89}	7.04×10^{-94}	−0.088	−0.22	−0.38
Fasting glucose[a]	0.66	2.33×10^{-3}	1.17×10^{-32}	1.77×10^{-34}	−0.058	−0.21	−0.23
HOMA-IR[a]	0.77	8.62×10^{-7}	1.78×10^{-96}	5.00×10^{-102}	−0.094	−0.22	−0.39
Fasting triglyceride[a]	0.89	1.74×10^{-5}	2.92×10^{-61}	4.48×10^{-85}	−0.082	−0.22	−0.36
BMI	0.09	3.53×10^{-7}	4.78×10^{-69}	1.55×10^{-75}	−0.097	−0.22	−0.31
			B. Adjustment of age, sex, and BMI				
Fasting insulin[a]	0.40	0.015	6.73×10^{-43}	1.77×10^{-44}	−0.047	−0.22	−0.26
Fasting glucose[a]	0.98	0.036	7.12×10^{-18}	1.03×10^{-18}	−0.040	−0.22	−0.17
HOMA-IR[a]	0.46	0.0053	3.16×10^{-49}	4.07×10^{-51}	−0.053	−0.23	−0.28
Fasting triglyceride[a]	0.73	0.00074	7.01×10^{-56}	3.78×10^{-58}	−0.065	−0.23	−0.30

[a]Calculated by using logarithm. rs12:rs12051271. a v, additive variable; pl adipo, plasma adiponectin; adj, adjustment; betw, between.

each metabolic phenotype and the rs12051272 genotypes (additive model) was −0.094 ($P = 8.62 \times 10^{-7}$), −0.088 ($P = 4.05 \times 10^{-6}$), −0.058 ($P = 2.33 \times 10^{-3}$), and −0.082 ($P = 1.74 \times 10^{-5}$), respectively. The results were consistent if BMI was included as an explanatory variable (Table 5B). This means the SNP genotype has a statistically significant negative effect on those phenotypes independent of plasma adiponectin levels, with rs12051272-G allele increasing these metabolic risks, but it is cancelled by its positive effect through increasing adiponectin levels simultaneously. The mechanism how the SNP genotype has effects on those phenotypes independent of plasma adiponectin levels remains to be elucidated.

From the other point of view, when we analyzed the effect of plasma adiponectin concentration on those phenotypes, the association became even stronger when adjusted for rs12051272 genotypes (Table 5A, B). That suggests when we use the plasma adiponectin levels as a marker for risk of cardiovascular events or MetS in the clinical practice, as proposed by several researchers, the SNP genotypes of *CDH13* gene have to be considered. Based on our results that SNPs for *CDH13* gene were strongly associated with HMW adiponictin, we tried to adjust HMW adiponectin instead of plasma adiponectin, but we only observed similar results between adjusting HMW adiponectin and plasma adiponectin levels (data not shown).

During preparation of this manuscript, a genome-wide association study in Korean population as well as in Filipino women reported that rs3865188:A>T in *CDH13* was indeed associated with adiponectin levels [Jee et al., 2010; Wu et al., 2010] as shown here. Also, it showed that G variant at rs12444338:G>T in linkage disequilibrium with rs3865188 had an increased promoter activity of *CDH13* gene in vitro [Jee et al., 2010]. However, our results did show that rs12051272 SNP genotypes were more significantly associated with adiponectin levels than rs12444338 SNP genotypes (Table 1 and Supp. Table S4), while rs12444338 SNP genotypes were in linkage disequilibrium with rs4783244 and rs12051272 (Supp. Table S5).

Discussion

Adiponectin is an adipokine, secreted specifically and abundantly by adipose tissues. It has attracted much attention because of its antidiabetic and antiatherosclerotic effects by sensitizing the body to insulin. It has been shown that adiponectin inhibits hepatic glucose production, enhances glucose uptake in muscle, increases fatty acid oxidation in both liver and muscle, and augments energy expenditure by enhancing uncoupling of ATP generation in mitochondria in vitro. Numerous studies have shown that plasma adiponectin concentration correlates negatively with fasting plasma insulin levels and insulin resistance measures. Lower plasma levels of adiponectin have been shown to be associated with obesity, type 2 diabetes, coronary artery disease, hyperlipidemia, hypertension, and the MetS. Some investigators suggest that it can be considered as a biomarker or a diagnostic marker to predict vulnerability for MetS [Martin et al., 2005; Santaniemi et al., 2006], cardiovascular events [Pischon et al., 2004], and in-stent restenosis after acute myocardial infarction [Kitta et al., 2008; Moldoveanu et al., 2008].

Adiponectin exists in three major oligomeric forms; a LMW trimer, a middle-molecular weight (MMW) hexamer, and HMW 12- to 18-mer. A small amount of globular form, possibly resulting from proteolytic cleavage, has also been described. Recently, HMW adiponectin has been especially attracting attention because the level of HMW adiponectin was reported to be more significantly associated with parameters related to glucose metabolism than other forms. The level of HMW adiponectin or the ratio of HMW to total adiponectin was shown to be more relevant to the prediction of insulin resistance [Lara-Castro et al., 2006].

T-cadherin (*CDH13*), which is expressed in endothelium and smooth muscle, has been identified as a receptor specific for HMW adiponectin [Hug et al., 2004]. The amino acid motif of T-cadherin is well conserved in higher eukaryote compared to that of E-cadherin, suggesting of some biological significance. It lacks a cytoplasmic domain and is anchored to the surface membrane via glycosyl phosphatidyl inositol (GPI) moiety, it is speculated that T-cadherin may act as a co-receptor along with other signaling molecules, but its physiological roles are largely unknown. T-cadherin has been shown to be more strongly expressed in regenerative endothelial cells and vascular smooth muscle cells in the region of atherosclerosis than in those of the normal artery, and the level of its expression is known to be correlated with the progression of atherosclerosis, implicating that it is playing some role in atherosclerotic changes.

We showed a single SNP rs12051272, tagging a corresponding haplotype constituting of rs12051272 and rs4783244, was significantly associated with the plasma adiponectin levels. The association of the rs12051272 SNP genotypes and plasma adiponectin levels was consistent across all studies. The effect size measured in eta squared in Suita study population suggested that the two variants rs12051272 in *CDH13* gene and Ile164Thr of *ADIPOQ* explained 3.28% and 2.62% of the variation in plasma adiponectin levels, respectively. Interaction between rs12051272 in the *CDH13* gene and +517T>C (Ile164Thr) within *ADIPOQ* gene was not statistically significant, though the combined effects of these two SNPs on plasma adiponectin concentrations were shown to be additive (data not shown). Although the rs12051272 showed the smaller

beta-coefficient than Ile164Thr, the contribution in the population was greater because of the higher MAF.

Previously, a similar GWAS study adiponectin was reported [Richards et al., 2009]. They used the same SNP platform, Illumina HumanHap550, as ours but failed to identify any *cis*- or *trans*-SNP of *CDH13* affecting the plasma adiponectin levels. Illumina HumanHap550 assay contains a probe for rs4783244 but not that for rs12051272. The haplotype structure data obtained from Human HapMap project showed that the MAF of rs4783244 was similarly high in both Caucasians and Japanese (0.46 and 0.32), while that of rs12051272 was much lower in Caucasians (0.01) than in Japanese (0.25). Based on our study results and the above, we estimated the power of detection for the SNP rs12051272 in Japanese as 0.992, while we did that in Caucasian as only 0.00002. Our result suggests that the haplotype constituting T allele of rs12051272 (G/T) and T allele of rs4783244 (G/T), tagged by a single rs12051272 SNP, has an effect in decreasing plasma adiponectin levels. Illumina HumanHap550 does not contain the probe for rs12051272, along with the too low MAF of this TT haplotype to be detected of significant association in Caucasian population that contains mostly GG and TG haplotypes, were the main reasons why GWAS in Caucasian population failed to identify the significant association. The difference of MAF may also partly explain the difference in the baseline plasma adiponectin levels between Japanese and American. Kadowaki et al. showed that the American men had been shown to have higher levels of adiponectin than the Japanese men despite higher levels of obesity [Kadowaki et al., 2006]. Since the majority of American had adiponectin-increasing rs12051272-G alleles, the mean plasma adiponectin levels can be apparently higher accordingly, but this does not mean the Americans are more resistant to risk phenotypes because this allele has an independent effect on augmenting insulin resistance as shown by the partial correlation coefficients. The mean difference in plasma adiponectin levels between GG and TT groups in Japanese was around 2 μg/ml consistently in both sexes in three independent groups, so the sole genotypic difference in T-cadherin gene may not explain the 6 μg/ml difference between Japanese and Americans.

Compared with the report of the genome-wide association study in Korean population as well as in Filipino women, our results did show that rs12051272 SNP genotypes were more significantly associated with adiponectin levels and especially with the levels of HMW adiponectin than rs12444338 SNP genotypes.

Our results provide two novel insights relating T-cadherin. First, in analyzing the physiological effects of plasma adiponectin, the analysis has to be adjusted for the SNP genotypes that would affect the 28% of SD changes in plasma adiponectin level per allele.

Several researchers have proposed to use the plasma adiponectin levels as a marker for risk of cardiovascular events or MetS in the clinical practice. For that purpose, the effect of the SNP genotypes, with the mean difference of 1 μg/ml per allele, on the levels of adiponectin is too large to ignore, especially in Japanese. Second, T-cadherin should have some unknown effect independent of adiponectin levels, since the same allele had an opposite effect judging from the results of partial correlation coefficient. The effect of the rs12051272 SNP also has to be elucidated. Since the SNP is located in the first intron of *CDH13* gene and the surrounding nucleotide sequence did not match the known transcription factor binding site or miRNA-targeted sequence, it is likely that the rs12051272 is a mere marker SNP tagging the phenotype-affecting haplotype present in 30-kb haplotype block covering from the promoter region to the first intron of CDH13. The idea that phenotype-affecting haplotype tagged by rs12051272 SNP would affect the baseline level of T-cadherin in the tissues is attractive, since the increasing amount of T-cadherin may capture the free adiponectin molecules in the plasma resulting in lowering plasma HMW adiponectin levels, simultaneously increasing the adiponectin signals in the peripheral vessel walls resulting in augmenting the effect of adiponectin per cell. Therefore, we may have to take the CHD13 genotypes as well as ADIPOQ genotypes into account if the plasma adiponectin levels is used as a marker for a risk of cardiovascular events or diabetes, though the attempt to compare the level of T-cadherin in peripheral vessel walls with the genotype will give us further evidence for this result.

Acknowledgments

We thank all those who participated in the study. In addition, we gratefully acknowledge all the members of Suita City Health Center and the Suita Medical Association. Also, we express our thanks to Ms. Yoko Miyamoto, and other members of the Department of Bioscience and Genetics for their technical support with this study.

This work was supported in part by a Grant for the Promotion of Fundamental Studies in Health Science from the Organization for Pharmaceutical Safety and Research (OPSR) of Japan, and a grant from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), as well as Research Grants for Cardiovascular Diseases from the Ministry of Health, Labour, and Welfare, Japan.

References

Heng D, Ma S, Lee JJ, Tai BC, Mak KH, Hughes K, Chew SK, Chia KS, Tan CE, Tai ES. 2006. Modification of the NCEP ATP III definitions of the metabolic syndrome for use in Asians identifies individuals at risk of ischemic heart disease. Atherosclerosis 186:367–373.

Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y. 2000. Plasma concentrations of a novel, adiposespecific protein, adiponectin, in type 2 diabetic patients. Arterioscler Thromb Vasc Biol 20:1595–1599.

Hug C, Wang J, Ahmad NS, Bogan JS, Tsao TS, Lodish HF. 2004. T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. Proc Natl Acad Sci USA 101:10308–10313.

Iwai N, Katsuya T, Mannami T, Higaki J, Ogihara T, Kokame K, Ogata J, Baba S. 2002. Association between *SAH*, an acyl-CoA synthetase gene, and hypertriglyceridemia, obesity, and hypertension. Circulation 105:41–47.

Jee SH, Sull JW, Lee JE, Shin C, Park J, Kimm H, Cho EY, Shin ES, Yun JE, Park JW, Kim SY, Lee SJ, Jee EJ, Baik I, Kao L, Yoon SK, Jang Y, Beaty TH. 2010. Adiponectin concentrations: a genome-wide association study. Am J Hum Genet 87:545–552.

Kadowaki T, Sekikawa A, Okamura T, Takamiya T, Kashiwagi A, Zaky WR, Maegawa H, El-Saed A, Nakamura Y, Evans RW, Edmundowicz D, Kita Y, Kuller LH, Ueshima H. 2006. Higher levels of adiponectin in American than in Japanese men despite obesity. Metabolism 55:1561–1563.

Kanazawa M, Yoshiike N, Osaka T, Numba Y, Zimmet P, Inoue S. 2002. Criteria and classification of obesity in Japan and Asia-Oceania. Asia Pac J Clin Nutr 11(Suppl 8):S732–S737.

Kern PA, Di Gregorio GB, Lu T, Rassouli N, Ranganathan G. 2003. Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression. Diabetes 52:1779–1785.

Kita Y, Takano H, Nakamura T, Kodama Y, Umetani K, Fujioka D, Saito Y, Kawabata K, Obata JE, Mende A, Kobayashi T, Kugiyama K. 2008. Low adiponectin levels predict late in-stent restenosis after bare metal stenting in native coronary arteries. Int J Cardiol 131:78–82.

Lara-Castro C, Luo N, Wallace P, Klein RL, Garvey WT. 2006. Adiponectin multimeric complexes and the metabolic syndrome trait cluster. Diabetes 55:249–259.

Lindsay RS, Funahashi T, Hanson RL, Matsuzawa Y, Tanaka S, Tataranni PA, Knowler WC, Krakoff J. 2002. Adiponectin and development of type 2 diabetes in the Pima Indian population. Lancet 360:57–58.

Ling H, Waterworth DM, Stirnadel HA, Pollin TI, Barter PJ, Kesäniemi YA, Mahley RW, McPherson R, Waeber G, Bersot TP, Cohen JC, Grundy SM, Mooser VE, Mitchell BD. 2009. Genome-wide linkage and association analyses to identify genes influencing adiponectin levels: the GEMS Study. Obesity 17:737–744.

Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, Matsubara K. 1996. cDNA cloning and expression of a novel adipose specific collagen-like factor,